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NUCLEIC ACID ENCODING PROTEINS INVOLVED IN PROTEIN DEGRADATION, PRODUCTS AND METHODS RELATED THERETO

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NUCLEIC ACID ENCODING PROTEINS INVOLVED IN PROTEIN DEGRADATION, PRODUCTS AND METHODS RELATED THERETO

This application is a continuation-in-part of application serial No. 09/591,694, filed June 9, 2000, which claims the benefit of U.S. Provisional Application No. 60/367,334, filed June 11, 1999, which was converted from U.S. Serial No. 09/330,517, and is incorporated herein by reference.

Portions of the invention described herein were made in the course of research supported in part by NIH grant CA67329. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to nucleic acids and proteins encoded thereby.

BACKGROUND OF THE INVENTION

The temporal coordination of sequential steps within the eukaryotic cell cycle is governed in large part by protein degradation, involving targeted ubiquitination of specific cell cycle regulatory proteins followed by their destruction by the 26S proteasome (reviewed in Ciechanover, A. 1998, EMBO J., 17(24):7151-7160). Among the cell cycle regulators whose levels are controlled by ubiquitination and subsequent proteosome-dependent degradation are the cyclins (cyclins A, B, C, D1, E) and several of the cyclin-dependent kinase (cdk) inhibitory proteins including p21-Waf1 and p27 Kip. Defects in this highly regulated process of protein turnover have been documented in many types of cancer.

The steps involved in polyubiquitination of specific proteins in cells involve the concerted actions of E1, E2, and E3-type enzymes. E1 proteins form thioester bonds in which the sulfhydryl group of internal cysteine residues binds the carboxyl amino acid of ubiquitin, thereby activating ubiquitin for subsequent transfer to E2-family proteins. E2 family proteins then transfer activated ubiquitin to the free amino-groups of lysine side chains in target proteins directly. More often, however, E2-family proteins collaborate with E3 proteins which bind particular target proteins and orchestrate their interactions with E2s, coordinating the polyubiquitination of these target proteins in highly regulated manners Ciechanover, A. 1998 supra. E3 functions are sometimes embodied in multiprotein complexes rather than mediated by a single protein.

The ubiquitination and degradation of a variety of cyclins, cyclin-dependent kinases (cdks) and cdk-inhibitors is temporally controlled during the cell cycle by SFC complexes. Theses multiprotein complexes function as E3-like entities, and contain the Skp-1 protein, at least one Cullin-family protein, and at least one F-box protein, thus the acronym SCF: S=Skp1; C=Cullin; F=F-box) (reviewed in Patton, E.E. et al., 1998, TIG 14(6):236-243). F-box proteins contain a conserved motif, the F-box, which mediates their interactions with Skp-1. The F-box proteins also contain other domains which allow them to simultaneously bind specific substrate proteins, which are then targeted for degradation via polyubiquitination. One such F-box protein identified in humans is b-Trcp, which forms a SCF complex with Skp-1 and Cul-1, and which interacts with β-catenin, targeting it for degradation (Latres, et al., 1999, Oncogene,

18:849-854, and Winston, J.J. et al., 1999, Genes & Dev., 13:270-283).

Siah-family proteins represent mammalian homologs of the Drosophila Sina protein. Sina is required for R7 photoreceptor cell differentiation within the sevenless pathway. Sina binds a ubiquitin-conjugating enzyme (E2) via an N-terminal RING domain. Heterocomplexes of Sina and another protein called Phyllopodia form a E3-complex which interacts with a transcriptional repressor called Tramtrack, targeting it for polyubiqitination and proteosome-mediated degradation in the fly (Tang, A.H. et al., 1997, Cell, 90:459-467 and Li, S. et al., 1997, Cell, 469-478). The destruction of Tramtrack is necessary for differentiation of R7 cells.

At present, little is known about the expression of mammalian genes related to the Siah-mediated-protein-degradation family of proteins in normal cells and cancers. Moreover, the diversity of functions of the Siah-mediated-protein-degradation family proteins remain unclear. Therefore, there continues to be a need in the art for the discovery of additional proteins that interact with the Siah-mediated-protein-degradation pathway, such as proteins that bind Siah in vivo, and especially a need for information serving to specifically identify and characterize such proteins in terms of their amino acid sequence. Moreover, to the extent that such molecules might form the basis for the development of therapeutic and diagnostic agents, it is essential that the DNA encoding them be elucidated. Similarly, a need exists to identify additional components of SCF complexes which may operate in concert with or independently of Siah. The present invention satisfies these needs and provides related advantages as well.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided novel isolated nucleic acids encoding a variety of Siah-Mediated-Degradation-Proteins (SMDPs) involved in the Siah-mediated protein degradation pathways and/or SCF-Complex-Proteins (SCPs) involved in SCF-mediated protein degradation pathways. Further provided are vectors containing invention nucleic acids, probes that hybridize thereto, host cells transformed therewith, antisense-nucleic acids thereto and related compositions. The nucleic acid molecules described herein can be incorporated into a variety of expression systems known to those of skill in the art. In addition, the nucleic acid molecules of the present invention are useful as probes for assaying for the presence and/or amount of a SMDP and/or SCP gene or mRNA transcript in a given sample. The nucleic acid molecules described herein, and oligonucleotide fragments thereof, are also useful as primers and/or templates in a PCR reaction for amplifying genes encoding SMDP and/or SCP proteins.

In accordance with the present invention, there are also provided isolated mammalian SMDP and/or SCP proteins. These proteins, or fragments thereof, are useful in bioassays, as immunogens for producing anti-SMDP and/or SCP antibodies, or in therapeutic compositions containing such proteins and/or antibodies. Also provided are transgenic non-human mammals that express, or fail to express (e.g., knock-out), the invention protein.

Antibodies that are immunoreactive with invention SMDP and/or SCP proteins are also provided. These antibodies are useful in diagnostic assays to

determine levels of SMDP and/or SCP proteins present in a given sample, e.g., tissue samples, Western blots, and the like. The antibodies can also be used to purify SMDP and/or SCP proteins from crude cell extracts and the like. Moreover, these antibodies are considered therapeutically useful to modulate the biological effect of SMDP and/or SCP proteins in vivo.

Also provided are bioassays for identifying compounds that modulate the activity of invention SMDP and/or SCP proteins. Methods and diagnostic systems for determining the levels of SMDP and/or SCP protein in various tissue samples are also provided. These diagnostic methods can be used for monitoring the level of therapeutically administered SMDP and/or SCP or fragments thereof to facilitate the maintenance of therapeutically effective amounts. These diagnostic methods can also be used to diagnose physiological disorders that result from abnormal levels of SMDP and/or SCP.

Also provided are systems using invention SMDPs, SCPs, or functional fragments thereof, or other protein-degradation binding domains, for targeting any desired protein for ubiquitination and degradation, thus enabling novel gene discovery through functional genomics strategies or providing the basis for ablating target proteins involved in diseases for therapeutic purposes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an amino acid sequence comparison of SIP-L (SEQ ID NO:4) and SIP-S (SEQ ID NO:6).

Figure 2 shows the Destruction-box amino acid consensus sequence of SAD.

Figure 3 shows the Mapping of Siah-APC interaction domains as described in Example 12.

Figure 4 shows the results of the cell proliferation functional assay of SIP/Siah interaction described in Example 7.

Figures 5A and 5B show in vitro and in vivo interaction assays of between Siah-1 and SIP-L as described in Examples 8 and 9, respectively.

Figures 6A and 6B show the mapping of SKP1, SIP-L, SAF-1 and SAD interaction domains as described in Example 13.

Figure 7 shows the effect of Siah-1 overexpression on stability of β-catenin.

Figure 8 shows a diagram of how an invention SIP communicates with the Protein Ubiquitination Machinery.

Figure 9 shows a general diagram of an invention method for inducing targeted degradation of proteins using SIP, exemplified in Example 15.

Figure 10 shows the results of the SIP-mediated degradation of the target TRAF6 protein, set forth in Example 15.

Figure 11A shows a model for Antizyme-dependent targeted protein degradation by ODC-conjugated proteins.

Figure 11B shows the amino acid sequence of an ODC-fusion protein (SEQ ID NO:50).

Figure 12 shows targeted degradation of TRAF6. HEK293T cells were transiently transfected with plasmids encoding HA-TRAF6, HA-TRAF2, ODC, ODC-TRAF6C, ODC-RANKpeptide, ODC-CD40CT or myc-Antizyme in various combinations, as indicated. After 24 h, cell lysates were prepared and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using antibodies specific for hemaglutinin (HA) (TRAF6) or Myc (ODC or Antizyme). The levels of TRAF6 mRNA were measured by Northern blot (mRNA).

Figure 13 shows additional examples of ODC-adapter-induced degradation of target protein(s). Figure 13 A shows antizyme-dependent targeted degradation of retinoblastoma (Rb) by ODC-E7 peptide. HEK293T cells were transiently transfected with plasmid encoding HA-Rb, ODC, ODC-E7 peptide or myc-Antizyme in various combinations, as indicated. Figure 13B shows antizyme-independent targeted degradation of Cdk2 by ODC-p21waf-1. HEK293T cells were transiently transfected with plasmid encoding myc-Cdk2, ODC, ODC-p21waf-1 or myc-Antizyme in various combinations, as indicated. Figure 13C shows antizyme-independent targeted degradation of IKKβ by ODC-IKKβ (leucine-zipper domain). HEK293T cells were transiently transfected with plasmid encoding HA-IKKβ, ODC, ODC-IKKβ-LZ or myc-Antizyme in various combinations, as indicated. After 24 h, cell lysates were prepared and analyzed by SDS-PAGE and immunoblotted using antibodies specific for Rb, Cdk2, IKKβ or HSC70 (as a control).

Figure 14 shows analysis of interactions of ODC-TRAF6C and TRAF6. HEK293T cells were transiently transfected with plasmids encoding hemaglutinin (HA)-tagged TRAF6 and ODC, ODC-TRAF6C peptide or Antizyme in various combinations, as indicated. After 24 h, 10 μM MG132 was added into culture media, and the cells were incubated another 6 hours. Lysates were subjected to immunoprecipitation using anti-myc monoclonal antibody-conjugated beads. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using an anti-HA monoclonal antibody with ECL-based detection. As a control, 0.1 volume of input cell lysate was loaded directly in the same gel (Input).

Figure 15 shows that targeted degradation of TRAF6 by ODC-RANK peptide is proteasome-dependent. HEK293T cells were transiently transfected with plasmid encoding HA-TRAF6, ODC-RANK peptide or myc-Antizyme in various combinations, as indicated. After 24 h, cells were either untreated, treated with 1 μM MG132 (MG132), 1 nM Epoximycine (Epox.), 10 μM Lactastacine (Lact.) or 1 μM Trypsin inhibitor (Tryp.) for 6 hours. Cell lysates were prepared and analyzed by SDS-PAGE and immunoblotted using antibodies specific for HA.

Figure 16 shows pulse-chase analysis of ectopically expressed HA-tagged TRAF6. HEK239T cells were transiently co-transfected with plasmids encoding HA-TRAF6 and ODC-RANK peptide, with or without myc-Antizyme. After 24 hours, cells were pulse-labeled with ^{35}S-methionine and cysteine, and then chased with media lacking the labeled amino acids. Cells were lysed at the indicated times, and the expressed HA-TRAF6 was recovered by immunoprecipitation via a HA epitope tag. Immunoprecipitated HA-TRAF6 was subjected to SDS-PAGE and

dried gels were analyzed with a PhosphorImager. Data from pulse-chase analysis is presented as the average ± SD from duplicate experiments.

Figure 17 shows functional analysis using ODC-E7 peptide. Figure 17A shows degradation of endogenous Rb protein by ODC-E7. HEK293T cells were transiently transfected with plasmid encoding ODC, ODC-E7 peptide or myc-Antizyme in various combinations, as indicated. After 48 h, lysates were prepared and subjected to immunoprecipitation using anti-Rb monoclonal antibody. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using an anti-Rb monoclonal antibody. Figure 17B shows the effect of ODC-E7 on E2F reporter activity. HEK293T cells were transiently transfected with a reporter gene plasmid that contains a E2F responsive element cloned upstream of a luciferase reporter gene, together with pCMVβ-gal as a transfection-efficiency control, and plasmids encoding ODC, ODC-E7 or Antizyme in various combinations, as indicated in Figure 17A. Luciferase activity was measured in cell lysates 24 hr later, and normalized relative to β-galactosidase (mean ± std. dev.; n = 3).

Figure 18 shows the effect of ODC-RANK peptide and ODC-TRAF6C on IL-1-induced NFκB reporter activity. HEK293T cells were transiently transfected with a reporter gene plasmid that contains an NFκB responsive element cloned upstream of a luciferase reporter gene, together with pCMVβ-gal as a transfection-efficiency control, and plasmids encoding ODC, ODC-TRAF6C, ODC-RANK peptide, or Antizyme in various combinations, as indicated. After 24 hours, cells were treated with 50 ng/ml IL-1 or 10 ng/ml TNFα for an additional 24 hours. Luciferase activity was measured in cell lysates and

normalized relative to β-galactosidase (mean ± std. dev.; n = 3).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated nucleic acids, which encode novel mammalian Siah-Mediated-Degradation-Proteins (SMDPs) and/or SCF-Complex-Proteins (SCPs), and functional fragments thereof. SMDPs are involved in the Siah-mediated protein degradation pathways and SCPs are involved in SCF-mediated protein degradation pathways. In some instances, these two pathways for protein degradation may operate in collaboration, particularly in cases where proteins have been identified that physically link SMDPs to SCPs. Invention SMDPs and/or SCPs are contemplated herein to regulate protein degradation, either by activating or inhibiting such protein degradation.

As used herein, invention SMDPs are proteins that participate in the Siah-mediated protein degradation pathway. The term "Siah" refers to the mammalian family of proteins encoded by at least two genes referred to as *SIAH1* and *SIAH2* (Hu, G. et al., 1997, Genomics, 46:103-111). Like their Drosophila counterpart protein Sina, the Siah-1 and Siah-2 proteins bind ubiquitin conjugating enzymes (UBCs) via an N-terminal RING domain and target other proteins for degradation.

As used herein, invention SCPs are proteins that participate in the Skp-1, Cullin, F-box (SCF) protein degradation pathway. These proteins can be components of SCF complexes or proteins that associate with SCF complexes. In some instances an invention

protein may fulfill the requirements of both a SMDP and a SCP.

Using yeast two-hybrid screening methods, targets of Siah-mediated protein degradation have been identified demonstrating the involvement of Siah and other inventions SMDPs and/or SCPs in pathways involved in cell growth regulation in cancers. For example, evidence is provided herein demonstrating that Siah-1 interacts indirectly with SCF complexes through associations with an invention SIP protein. Siah-1 has also been found to be an important regulator of cell growth, through its effects on ubiquitination and degradation of β-catenin and possibly other target proteins including an invention protein SAD.

Thus far, the only reported target of Siah-mediated degradation is DCC (Hu, G. et al., 1997, Genes & Dev., 11:2701-2714), a putative tumor suppressor protein encoded by a gene which is commonly disrupted in colon cancers (Fearon, E.R. et al., 1990, Science, 247:49-56). The involvement of DCC in colon cancers however has recently been questioned, and it appears that a different gene located near DCC on 18q21 is the primary target of deletions in this chromosomal region (Fearon, E.R. et al., 1990, supra). However, the DCC protein has recently been shown to deliver either pro-apoptotic or anti-apoptotic signals, depending on whether it is complexed with its ligand Netrin. These observations suggest that deletion or inactivation of DCC could potentially contribute to tumorigensis by removing a pro-apoptotic influence from cells.

Another connection between the Siah-family of proteins and tumor suppressor genes has been found for

p53. For example, the Siah-1 gene of mice was found among a group of immediate-early genes induced by p53 using a hemopoietic cell line as a model for p53-induced cell cycle arrest and apoptosis (Amson, R.B. et al., 1996, PNAS, USA, 93:3953-3957). Expression of Siah-1 was also indirectly correlated with increased apoptosis in tumor xenograph experiments, suggesting that Siah-1 could function as a tumor suppressor in some contexts (Nemani, M. et al., 1996, Proc. Natl. Sci. USA 93:9039-9042).

It has also been found that Siah-1 over-expression can induce cell cycle arrest independently of apoptosis in epithelial cancer cells (Matsuzawa, et al., 1998, EMBO J., 17(10):2736-2747). Moreover, UV-irradiation at subapoptotic doses was shown to induce Siah-1 gene expression in MCF7 breast cancer cells and to promote cell cycle arrest. These and other data have implicated Siah-1 in a p53-inducible pathway for cell cycle arrest which runs parallel to the well-studied p21-Waf1 pathway (Matsuzawa, et al. 1998, supra).

The phrase "SMDP and/or SCP" refers to substantially pure native SMDP and/or SCP, or recombinantly produced proteins, including naturally occurring allelic variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, and further including fragments thereof which retain at least one native biological activity, such as immunogenicity, the ability to bind to a SMDP and/or SCP, and the like. Exemplary SMDPs and/or SCPs referred to herein include amino acid sequences set forth in SEQ ID Nos:2 (Siah-1α), 4 (SIP-L), 6 (SIP-S), 8 (SAF-1α), 10 (SAF-1β), 12 (SAF-2) and 14 (SAD). Invention isolated SMDPs and/or SCPs are substantially pure and free of

cellular components and/or contaminants normally associated with a native *in vivo* environment.

As used herein, the term "Siah-1α" refers to a splice-variant member of the mammalian, preferably human, Siah-family of proteins. The invention Siah-1α protein, or functional fragment thereof, is characterized by having the ability to bind to at least one or more of the proteins selected from APC (Kinzler K.W., et al., 1996, Cell, 87(2):159-170); BAG-1 (Takayama et al., 1995, Cell, 80(2):279-284); SIP-L; SIP-S; or other Siah proteins, such as Siah-1. Thus, homodimers of Siah-1α are contemplated herin. Invention Siah-1α proteins differ from Siah-1β (set forth as SIAH-1 in Hu et al., 1997, Genomics, 46:103-111) by containing an additional 16 amino acids at the amino-terminus. Thus, preferred invention Siah-1α proteins, and fragments thereof, comprise at least a portion of the 16 N-terminal amino acids of SEQ ID NO:2. A particularly preferred Siah-1α protein is set forth in SEQ ID NO:2.

In accordance with another embodiment of the invention, Siah-1 has been found to interact with an invention protein referred to herein as the "SIP" family. As used herein, the term "SIP" refers to any species, preferably mammalian, more preferably human, Siah-1-Interacting Protein (SIP). The invention SIP proteins, or functional fragments thereof, are characterized by having the ability to bind to at least one or more of the proteins selected from Siah-1, Skp1, or other SIP proteins. Thus, homodimers of invention SIP proteins are contemplated herein. The SIP gene has been found to encode at least two proteins through alternative mRNA splicing: SIP-L (L, for long; SEQ ID NO:4), and SIP-S (S, for short; SEQ ID NO:6). A sequence comparison of SIP-L

to SIP-S is set forth in Figure 1. To further identify potential targets of Siah-1-mediated ubiquitin/proteasome protein degradation, yeast two-hybrid screens of cDNA libraries were performed using the invention human SIP-L protein (SEQ ID NO:4) as a bait. Such screen resulted in the identification of Skp1 (Zhang et al., 1995, Cell, 82(6):915-925).

As shown in Figure 8, an invention SIP protein binds simultaneously to Siah and Skp, proteins known to bind directly or indirectly, respectively, to ubiquitin-conjugating enzymes (E2s). Therefore, in accordance with the present invention, this characteristic of SIP is useful for methods for targeting desired proteins for degradation, via a ubiquitin/proteosome-dependent mechanism (see, e.g., Example 15).

In accordance with another embodiment of the invention, using a yeast two-hybrid screen with Skp1 as bait (set forth in the Examples), two additional invention SMDP and/or SCP proteins were identified as Skp1-interacting proteins, which are referred to herein as SAF-1 and SAD. As used herein the term "SAF-1" refers to Skp1-Associated F-box protein-1. The invention SAF-1 proteins, or functional fragments thereof, are characterized by having the ability to bind to at least one or more of the proteins selected from Skp1, SIP, such as SIP-L, or SAD. Invention SAF-1 proteins are further characterized as containing an "F-box" amino acid domain. Exemplary SAF-1 proteins include SAF-1α (SEQ ID NO:8) and SAF-1β (SEQ ID NO:10). An exemplary F-box domain is set forth as amino acids 256-296 of SEQ ID NO:8 and amino acids 335-375 of SEQ ID NO:10(see Figure 6). SAF-1 beta has the same F-Box as alfa, the location is in amino acids 335-375. In accordance with the present invention,

a homologue of SAF-1 protein has also been identified in the NCBI BLAST data base (Human DNA sequence from clone 341E18 on chromosome 6p11.2-12.3, AL031178) which shares significant homology with F-box domain of SAF-1. The invention homolog is referred to herein as SAF-2 and is set forth in SEQ ID Nos:11 and 12.

As used herein the term "SAD" refers to Skp1-Associated Destruction-box protein. The invention SAF-1 proteins, or functional fragments thereof, are characterized by having the ability to bind to at least one or more of the proteins selected from Skp1, SIP, such as SIP-L, or SAF-1. It is also contemplated herein that SAD has the ability to bind to SAF-2. Invention SAD proteins are further characterized as containing an "D-box" (Destruction-box) amino acid domain. An exemplary SAD protein is set forth herein as SEQ ID NO:14. As used herein, the D-box domain comprises the consensus amino acid sequence -DSGX_1X_2S-, wherein X_1 is preferably selected from a hydrophobic amino acid, such as Y, I, L, M, F, W, or V; and X_2 is any amino acid (see Figure 2). A preferred D-box domain comprises the sequence set forth as amino acids 144-149 of SEQ ID NO:14.

Thus, exemplary functional fragments of an invention SAD protein comprise at least amino acids 144-149 of SEQ ID NO:14. Also contemplated herein are functional fragments of inventions SAD proteins that bind to a SIP protein, and preferably comprise at least amino acids 360-447 of SEQ ID NO:14. Functional fragments of inventions SAD proteins that bind to a Skp1 protein preferably comprise at least amino acids 128-359 of SEQ ID NO:14. Functional fragments of inventions SAD proteins that bind to a SAF-1 protein preferably comprise at least amino acids 1-127 of SEQ ID NO:14.

The nucleic acid molecules described herein are useful for producing invention proteins, when such nucleic acids are incorporated into a variety of protein expression systems known to those of skill in the art. In addition, such nucleic acid molecules or fragments thereof can be labeled with a readily detectable substituent and used as hybridization probes for assaying for the presence and/or amount of an invention SMDP and/or SCP gene or mRNA transcript in a given sample. The nucleic acid molecules described herein, and fragments thereof, are also useful as primers and/or templates in a PCR reaction for amplifying genes encoding invention proteins described herein.

The term "nucleic acid" (also referred to as polynucleotides) encompasses ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), probes, oligonucleotides, and primers. DNA can be either complementary DNA (cDNA) or genomic DNA, e.g. a gene encoding a SMDP and/or SCP. One means of isolating a nucleic acid encoding an SMDP and/or SCP polypeptide is to probe a mammalian genomic library with a natural or artificially designed DNA probe using methods well known in the art. DNA probes derived from the SMDP and/or SCP gene are particularly useful for this purpose. DNA and cDNA molecules that encode SMDP and/or SCP polypeptides can be used to obtain complementary genomic DNA, cDNA or RNA from mammalian (e.g., human, mouse, rat, rabbit, pig, and the like), or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries, by methods described in more detail below. Examples of nucleic acids are RNA, cDNA, or isolated genomic DNA encoding an SMDP and/or SCP polypeptide. Such nucleic acids may include, but are not limited to, nucleic acids comprising substantially the same

nucleotide sequence as set forth in SEQ ID Nos:1 (Siah-1α), 3 (SIP-L), 5 (SIP-S), 7 (SAF-1α), 9 (SAF-1β), 11 (SAF-2) and 13 (SAD).

Use of the terms "isolated" and/or "purified" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been produced in such form by the hand of man, and thus are separated from their native *in vivo* cellular environment, and are substantially free of any other species of nucleic acid or protein. As a result of this human intervention, the recombinant DNAs, RNAs, polypeptides and proteins of the invention are useful in ways described herein that the DNAs, RNAs, polypeptides or proteins as they naturally occur are not.

Invention proteins can be obtained from any species of organism, such as prokaryotes, eukaryotes, plants, fungi, vertebrates, invertebrates, and the like. A particular species can be d mammalian, As used herein, "mammalian" refers to a subset of species from which an invention SMDP and/or SCP is derived, e.g., human, rat, mouse, rabbit, monkey, baboon, bovine, porcine, ovine, canine, feline, and the like. A preferred SMDP and/or SCP herein, is human SMDP and/or SCP.

In one embodiment of the present invention, cDNAs encoding the invention SMDPs and/or SCPs disclosed herein comprise substantially the same nucleotide sequence as the coding region set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. Preferred cDNA molecules encoding the invention proteins comprise the same nucleotide sequence as as the coding region set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13.

As employed herein, the term "substantially the same nucleotide sequence" refers to DNA having sufficient identity to the reference polynucleotide, such that it will hybridize to the reference nucleotide under moderately stringent hybridization conditions. In one embodiment, DNA having substantially the same nucleotide sequence as the reference nucleotide sequence encodes substantially the same amino acid sequence as that set forth in any of SEQ ID Nos:2, 4, 6, 8, 10, 12 or 14. In another embodiment, DNA having "substantially the same nucleotide sequence" as the reference nucleotide sequence has at least 60% identity with respect to the reference nucleotide sequence. DNA having at least 70%, more preferably at least 90%, yet more preferably at least 95%, identity to the reference nucleotide sequence is preferred.

This invention also encompasses nucleic acids which differ from the nucleic acids shown in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13, but which have the same phenotype. Phenotypically similar nucleic acids are also referred to as "functionally equivalent nucleic acids". As used herein, the phrase "functionally equivalent nucleic acids" encompasses nucleic acids characterized by slight and non-consequential sequence variations that will function in substantially the same manner to produce the same protein product(s) as the nucleic acids disclosed herein. In particular, functionally equivalent nucleic acids encode polypeptides that are the same as those encoded by the nucleic acids disclosed herein or that have conservative amino acid variations. For example, conservative variations include substitution of a non-polar residue with another non-polar residue, or substitution of a charged residue with a similarly charged residue. These variations include those

recognized by skilled artisans as those that do not substantially alter the tertiary structure of the protein.

Further provided are nucleic acids encoding SMDP and/or SCP polypeptides that, by virtue of the degeneracy of the genetic code, do not necessarily hybridize to the invention nucleic acids under specified hybridization conditions. Preferred nucleic acids encoding the invention SMDPs and/or SCPs are comprised of nucleotides that encode substantially the same amino acid sequence as set forth in SEQ ID Nos:2, 4, 6, 8, 10, 12 or 14.

Thus, an exemplary nucleic acid encoding an invention SMDP and/or SCP may be selected from:

(a) DNA encoding the amino acid sequence set forth in SEQ ID Nos:2, 4, 6, 8, 10, 12 or 14,

(b) DNA that hybridizes to the DNA of (a) under moderately stringent conditions, wherein said DNA encodes biologically active SMDP and/or SCP, or

(c) DNA degenerate with respect to either (a) or (b) above, wherein said DNA encodes biologically active SMDP and/or SCP.

Hybridization refers to the binding of complementary strands of nucleic acid (i.e., sense:antisense strands or probe:target-DNA) to each other through hydrogen bonds, similar to the bonds that naturally occur in chromosomal DNA. Stringency levels used to hybridize a given probe with target-DNA can be readily varied by those of skill in the art.

The phrase "stringent hybridization" is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions.

As used herein, the phrase "moderately stringent hybridization" refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, more preferably about 85% identity to the target DNA; with greater than about 90% identity to target-DNA being especially preferred. Preferably, moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C.

The phrase "high stringency hybridization" refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C.

The phrase "low stringency hybridization" refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C. Denhart's solution and SSPE (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers.

As used herein, the term "degenerate" refers to codons that differ in at least one nucleotide from a reference nucleic acid, e.g., SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13, but encode the same amino acids as the reference nucleic acid. For example, codons specified by the triplets "UCU", "UCC", "UCA", and "UCG" are degenerate with respect to each other since all four of these codons encode the amino acid serine.

Preferred nucleic acids encoding the invention polypeptide(s) hybridize under moderately stringent, preferably high stringency, conditions to substantially the entire sequence, or substantial portions (i.e., typically at least 15-30 nucleotides) of the nucleic acid sequence set forth in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13.

The invention nucleic acids can be produced by a variety of methods well-known in the art, e.g., the methods described herein, employing PCR amplification using oligonucleotide primers from various regions of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13, and the like.

In accordance with a further embodiment of the present invention, optionally labeled SMDP and/or SCP-

encoding cDNAs, or fragments thereof, can be employed to probe library(ies) (e.g., cDNA, genomic, and the like) for additional nucleic acid sequences encoding novel mammalian SMDPs and/or SCPs. Construction of suitable mammalian cDNA libraries is well-known in the art. Screening of such a cDNA library is initially carried out under low-stringency conditions, which comprise a temperature of less than about 42°C, a formamide concentration of less than about 50%, and a moderate to low salt concentration.

Presently preferred probe-based screening conditions comprise a temperature of about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5X standard saline citrate (SSC; 20X SSC contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0). Such conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring perfect homology. The phrase "substantial similarity" refers to sequences which share at least 50% homology. Preferably, hybridization conditions will be selected which allow the identification of sequences having at least 70% homology with the probe, while discriminating against sequences which have a lower degree of homology with the probe. As a result, nucleic acids having substantially the same nucleotide sequence as SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13 are obtained.

As used herein, a nucleic acid "probe" is single-stranded DNA or RNA, or analogs thereof, that has a sequence of nucleotides that includes at least 14, at least 20, at least 50, at least 100, at least 200, at least 300, at least 400, or at least 500 contiguous bases that are the same as (or the complement of) any

contiguous bases set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. Preferred regions from which to construct probes include 5' and/or 3' coding regions of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. In addition, the entire cDNA encoding region of an invention SMDP and/or SCP, or the entire sequence corresponding to SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13, may be used as a probe. Probes may be labeled by methods well-known in the art, as described hereinafter, and used in various diagnostic kits.

It is understood that a SMDP and/or SCP-encoding nucleic acid molecule of the invention, as used herein, specifically excludes previously known nucleic acid molecules consisting of nucleotide sequences having identity with the SMDP and/or SCP-encoding nucleotide sequence (e.g., SEQ ID NO:NOs:1, 3, 5, 7, 9, 11, 13), such as Expressed Sequence Tags (ESTs), Sequence Tagged Sites (STSs) and genomic fragments, deposited in public databases such as the nr, dbest, dbsts, gss and htgs databases, which are available for searching at http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0, using the program BLASTN 2.0.9 described by Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997).

In particular, a SMDP and/or SCP-encoding nucleic acid molecule specifically excludes nucleic acid molecules consisting of any of the nucleotide sequences having the Genbank (gb), EMBL (emb) or DDBJ (dbj) accession numbers described below. Similarly, a SMDP and/or SCP polypeptide fragment specifically excludes the amino acid fragments encoded by the nucleotide sequences having the GenBank accession numbers described below. GenBank accession numbers specifically excluded include NCBI ID: AA054272, AA258606, AA923663, AA418482, and

AI167464. The human sequence referenced as GenBank accession No. AL031178 is also specifically excluded from an invention SMDP and/or SCP-encoding nucleic acid.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal. Any label or indicating means can be linked to invention nucleic acid probes, expressed proteins, polypeptide fragments, or antibody molecules. These atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry.

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturation to form a fluorochrome (dye) that is a useful immunofluorescent tracer. A description of immunofluorescent analytic techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In one embodiment, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, and the like. In another embodiment, radioactive elements are employed labeling agents. The linking of a label to a substrate, i.e., labeling of nucleic acid probes, antibodies, polypeptides, and proteins, is well known in the art. For instance, an invention antibody can be labeled by metabolic incorporation of radiolabeled amino acids provided in the culture medium. See, for example, Galfre et al., Meth.

Enzymol., **73**:3-46 (1981). Conventional means of protein conjugation or coupling by activated functional groups are particularly applicable. See, for example, Aurameas et al., Scand. J. Immunol., Vol. 8, Suppl. **7**:7-23 (1978), Rodwell et al., Biotech., **3**:889-894 (1984), and U.S. Patent No. 4,493,795.

In accordance with another embodiment of the present invention, there are provided isolated mammalian Siah-Mediated-Degradation-Proteins (SMDPs) and/or SCF-Complex-Proteins (SCPs), and fragments thereof encoded by invention nucleic acid. The phrase "SMDP and/or SCP" refers to substantially pure native SMDP and/or SCP, or recombinantly produced proteins, including naturally occurring allelic variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, and further including fragments thereof which retain at least one native biological activity, such as immunogenicity, the ability to bind to another member of the SMDP and/or SCP families, or to homodimerize. In another embodiment, SMDPs and/or SCPs referred to herein, are those polypeptides specifically recognized by an antibody that also specifically recognizes a SMDP and/or SCP (preferably human) including an amino acid sequence set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12 and 14. Invention isolated SMDPs and/or SCPs are substantially pure and free of cellular components and/or contaminants normally associated with a native *in vivo* environment.

Presently preferred SMDPs and/or SCPs of the invention include proteins that comprise substantially the same amino acid sequences as the protein sequence set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12 and 14, as well as biologically active, functional fragments thereof.

Those of skill in the art will recognize that numerous residues of the above-described sequences can be substituted with other, chemically, sterically and/or electronically similar residues without substantially altering the biological activity of the resulting receptor species. In addition, larger polypeptide sequences containing substantially the same sequence as amino acids set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12 and 14 therein (e.g., splice variants) are contemplated.

As employed herein, the term "substantially the same amino acid sequence" refers to amino acid sequences having at least about 70% identity with respect to the reference amino acid sequence, and retaining comparable functional and biological activity characteristic of the protein defined by the reference amino acid sequence. Preferably, proteins having "substantially the same amino acid sequence" will have at least about 80%, more preferably 90% amino acid identity with respect to the reference amino acid sequence; with greater than about 95% amino acid sequence identity being especially preferred. It is recognized, however, that polypeptides (or nucleic acids referred to hereinbefore) containing less than the described levels of sequence identity arising as splice variants or that are modified by conservative amino acid substitutions, or by substitution of degenerate codons are also encompassed within the scope of the present invention.

The term "biologically active" or "functional", when used herein as a modifier of invention SMDP and/or SCP(s), or polypeptide fragments thereof, refers to a polypeptide that exhibits functional characteristics similar to SMDP and/or SCP. For example, one biological activity of SMDP and/or SCP is the ability to bind,

preferably in vivo, to at least one other member of the SMDP and/or SCP families of proteins, or to homodimerize, or to mediate protein degradation via an SFC complex as described herein. Such SMDP and/or SCP binding activity can be assayed, for example, using the methods described herein. Another biological activity of SMDP and/or SCP is the ability to act as an immunogen for the production of polyclonal and monoclonal antibodies that bind specifically to an invention SMDP and/or SCP. Thus, an invention nucleic acid encoding SMDP and/or SCP will encode a polypeptide specifically recognized by an antibody that also specifically recognizes the SMDP and/or SCP protein (preferably human) including the amino acid set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12 and 14. Such immunologic activity may be assayed by any method known to those of skill in the art. For example, a test-polypeptide encoded by a SMDP and/or SCP cDNA can be used to produce antibodies, which are then assayed for their ability to bind to an invention SMDP and/or SCP protein including the sequence set forth in SEQ ID Nos:2, 4, 6, 8, 10, 12 or 14. If the antibody binds to the test-polypeptide and the protein including the sequence encoded by SEQ ID NOs:2, 4, 6, 8, 10, 12 or 14 with substantially the same affinity, then the polypeptide possesses the requisite immunologic biological activity.

The invention SMDPs and/or SCPs can be isolated by a variety of methods well-known in the art, e.g., recombinant expression systems described herein, precipitation, gel filtration, ion-exchange, reverse-phase and affinity chromatography, and the like. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. **182**, (Academic Press, (1990)), which is incorporated herein by reference. Alternatively, the isolated

polypeptides of the present invention can be obtained using well-known recombinant methods as described, for example, in Sambrook et al., supra., 1989).

An example of the means for preparing the invention polypeptide(s) is to express nucleic acids encoding the SMDP and/or SCP in a suitable host cell, such as a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), or a mammalian cell, using methods well known in the art, and recovering the expressed polypeptide, again using well-known methods. Invention polypeptides can be isolated directly from cells that have been transformed with expression vectors as described below herein. The invention polypeptide, biologically functional fragments, and functional equivalents thereof can also be produced by chemical synthesis. For example, synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

Also encompassed by the term SMDP and/or SCP are functional fragments or polypeptide analogs thereof. The term "functional fragment" refers to a peptide fragment that is a portion of a full length SMDP and/or SCP protein, provided that the portion has a biological activity, as defined above, that is characteristic of the corresponding full length protein. For example, a functional fragment of an invention SMDP and/or SCP protein can have the protein:protein binding activity prevalent in SMDPs and/or SCPs. In addition, the characteristic of a functional fragment of invention SMDP and/or SCP proteins to elicit an immune response is useful for obtaining an anti-SMDP and/or SCP antibodies. Thus, the invention also provides functional fragments of

invention SMDP and/or SCP proteins, which can be identified using the binding and routine methods, such as bioassays described herein.

The term "polypeptide analog" includes any polypeptide having an amino acid residue sequence substantially the same as a sequence specifically shown herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to functionally mimic an SMDP and/or SCP as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The amino acid length of functional fragments or polypeptide anlogs of the present invention can range from about 5 amino acids up to the full-length protein sequence of an invention SMDP and/or SCP. In certain embodiments, the amino acid lengths include, for example, at least about 10 amino acids, at least about 20, at least about 30, at least about 40, at least about 50, at least about 75, at least about 100, at least about 150, at least about 200, at least about 250 or more amino acids in length up to the full-length SMDP and/or SCP protein sequence.

As used herein the phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue, provided that such polypeptide displays the required binding activity. The phrase "chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions of residues, relative to the sequence of a polypeptide whose sequence is shown herein, so long as the required activity is maintained.

The present invention also provides compositions containing an acceptable carrier and any of an isolated, purified SMDP and/or SCP mature protein or functional polypeptide fragments thereof, alone or in

combination with each other. These polypeptides or proteins can be recombinantly derived, chemically synthesized or purified from native sources. As used herein, the term "acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The SMDP and/or SCP compositions described herein can be used, for example, in methods described hereinafter.

Also provided are antisense-nucleic acids having a sequence capable of binding specifically with full-length or any portion of an mRNA that encodes SMDP and/or SCP polypeptides so as to prevent translation of the mRNA. The antisense-nucleic acid may have a sequence capable of binding specifically with any portion of the sequence of the cDNA encoding SMDP and/or SCP polypeptides. As used herein, the phrase "binding specifically" encompasses the ability of a nucleic acid sequence to recognize a complementary nucleic acid sequence and to form double-helical segments therewith via the formation of hydrogen bonds between the complementary base pairs. An example of an antisense-nucleic acid is an antisense-nucleic acid comprising chemical analogs of nucleotides.

Compositions comprising an amount of the antisense-nucleic acid, described above, effective to reduce expression of SMDP and/or SCP polypeptides by passing through a cell membrane and binding specifically with mRNA encoding SMDP and/or SCP polypeptides so as to prevent translation and an acceptable hydrophobic carrier capable of passing through a cell membrane are also provided herein. Suitable hydrophobic carriers are

described, for example, in U.S. Patent Nos. 5,334,761; 4,889,953; 4,897,355, and the like. The acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a receptor specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind to a cell-type specific receptor.

Antisense-nucleic acid compositions are useful to inhibit translation of mRNA encoding invention polypeptides. Synthetic oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding SMDP and/or SCP polypeptides and inhibit translation of mRNA and are useful as compositions to inhibit expression of SMDP and/or SCP associated genes in a tissue sample or in a subject.

In accordance with another embodiment of the invention, kits are provided for detecting mutations, duplications, deletions, rearrangements and aneuploidies in SMDP and/or SCP genes comprising at least one invention probe or antisense nucleotide.

The present invention provides means to modulate levels of expression of SMDP and/or SCP polypeptides by employing synthetic antisense-nucleic acid compositions (hereinafter SANC) which inhibit translation of mRNA encoding these polypeptides. Synthetic oligonucleotides, or other antisense-nucleic acid chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to full-length or portions of an SMDP and/or SCP coding strand, including nucleotide sequences set forth in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. The

SANC is designed to be stable in the blood stream for administration to a subject by injection, or in laboratory cell culture conditions. The SANC is designed to be capable of passing through the cell membrane in order to enter the cytoplasm of the cell by virtue of physical and chemical properties of the SANC which render it capable of passing through cell membranes, for example, by designing small, hydrophobic SANC chemical structures, or by virtue of specific transport systems in the cell which recognize and transport the SANC into the cell. In addition, the SANC can be designed for administration only to certain selected cell populations by targeting the SANC to be recognized by specific cellular uptake mechanisms which bind and take up the SANC only within select cell populations. In a particular embodiment the SANC is an antisense oligonucleotide.

For example, the SANC may be designed to bind to a receptor found only in a certain cell type, as discussed *supra*. The SANC is also designed to recognize and selectively bind to target mRNA sequence, which may correspond to a sequence contained within the sequences shown in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. The SANC is designed to inactivate target mRNA sequence by either binding thereto and inducing degradation of the mRNA by, for example, RNase I digestion, or inhibiting translation of mRNA target sequence by interfering with the binding of translation-regulating factors or ribosomes, or inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups which either degrade or chemically modify the target mRNA. SANCs have been shown to be capable of such properties when directed against mRNA targets (see Cohen et al., TIPS, **10**:435

(1989) and Weintraub, Sci. American, January (1990), pp.40; both incorporated herein by reference).

In accordance with yet another embodiment of the present invention, there is provided a method for the recombinant production of invention SMDPs and/or SCPs by expressing the above-described nucleic acid sequences in suitable host cells. Recombinant DNA expression systems that are suitable to produce SMDPs and/or SCPs described herein are well-known in the art. For example, the above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof.

Suitable expression vectors are well-known in the art, and include vectors capable of expressing DNA operatively linked to a regulatory sequence, such as a promoter region that is capable of regulating expression of such DNA. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the inserted DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in

the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Prokaryotic transformation vectors are well-known in the art and include pBlueskript and phage Lambda ZAP vectors (Stratagene, La Jolla, CA), and the like. Other suitable vectors and promoters are disclosed in detail in U.S. Patent No. 4,798,885, issued January 17, 1989, the disclosure of which is incorporated herein by reference in its entirety.

Other suitable vectors for transformation of *E. coli* cells include the pET expression vectors (Novagen, see U.S patent 4,952,496), e.g., pET11a, which contains the T7 promoter, T7 terminator, the inducible *E. coli* lac operator, and the lac repressor gene; and pET 12a-c, which contain the T7 promoter, T7 terminator, and the *E. coli* ompT secretion signal. Another suitable vector is the pIN-IIIompA2 (see Duffaud et al., Meth. in Enzymology, **153**:492-507, 1987), which contains the lpp promoter, the lacUV5 promoter operator, the ompA secretion signal, and the lac repressor gene.

Exemplary, eukaryotic transformation vectors, include the cloned bovine papilloma virus genome, the cloned genomes of the murine retroviruses, and eukaryotic cassettes, such as the pSV-2 gpt system [described by Mulligan and Berg, Nature Vol. **277**:108-114 (1979)] the Okayama-Berg cloning system [Mol. Cell Biol. Vol. **2**:161-170 (1982)], and the expression cloning vector described by Genetics Institute [Science Vol. **228**:810-815 (1985)], are available which provide substantial assurance of at least some expression of the protein of interest in the transformed eukaryotic cell line.

In accordance with another embodiment of the present invention, there are provided "recombinant cells" containing the nucleic acid molecules (i.e., DNA or mRNA) of the present invention. Methods of transforming suitable host cells, preferably bacterial cells, and more preferably *E. coli* cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989).

Exemplary methods of transformation include, e.g., transformation employing plasmids, viral, or bacterial phage vectors, transfection, electroporation, lipofection, and the like. The heterologous DNA can optionally include sequences which allow for its extrachromosomal maintenance, or said heterologous DNA can be caused to integrate into the genome of the host (as an alternative means to ensure stable maintenance in the host).

Host organisms contemplated for use in the practice of the present invention include those organisms in which recombinant production of heterologous proteins has been carried out. Examples of such host organisms include bacteria (e.g., *E. coli*), yeast (e.g., *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha* and *P. pastoris*; see, e.g., U.S. Patent Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), mammalian cells (e.g., HEK293, CHO and Ltk$^-$ cells), insect cells, and the like. Presently preferred host organisms are bacteria. The most preferred bacteria is *E. coli*.

In one embodiment, nucleic acids encoding the invention SMDPs and/or SCPs can be delivered into mammalian cells, either <u>in vivo</u> or <u>in vitro</u> using suitable viral vectors well-known in the art. Suitable retroviral vectors, designed specifically for "gene therapy" methods, are described, for example, in WIPO publications WO 9205266 and WO 9214829, which provide a description of methods for efficiently introducing nucleic acids into human cells. In addition, where it is desirable to limit or reduce the <u>in vivo</u> expression of the invention SMDP and/or SCP, the introduction of the antisense strand of the invention nucleic acid is contemplated.

Viral based systems provide the advantage of being able to introduce relatively high levels of the heterologous nucleic acid into a variety of cells. Suitable viral vectors for introducing invention nucleic acid encoding an SMDP and/or SCP protein into mammalian cells (e.g., vascular tissue segments) are well known in the art. These viral vectors include, for example, Herpes simplex virus vectors (e.g., Geller et al., <u>Science</u>, **241**:1667-1669 (1988)), Vaccinia virus vectors

(e.g., Piccini et al., Meth. in Enzymology, **153**:545-563 (1987); Cytomegalovirus vectors (Mocarski et al., in Viral Vectors, Y. Gluzman and S.H. Hughes, Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988, pp. 78-84), Moloney murine leukemia virus vectors (Danos et_al., PNAS, USA, **85**:6469 (1980)), adenovirus vectors (e.g., Logan et al., PNAS, USA, **81**:3655-3659 (1984); Jones et al., Cell, **17**:683-689 (1979); Berkner, Biotechniques, **6**:616-626 (1988); Cotten et al., PNAS, USA, **89**:6094-6098 (1992); Graham et al., Meth. Mol. Biol., **7**:109-127 (1991)), adeno-associated virus vectors, retrovirus vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), and the like. Especially preferred viral vectors are the adenovirus and retroviral vectors.

For example, in one embodiment of the present invention, adenovirus-transferrin/polylysine-DNA (TfAdpl-DNA) vector complexes (Wagner et al., PNAS, USA, **89**:6099-6103 (1992); Curiel et al., Hum. Gene Ther., **3**:147-154 (1992); Gao et al., Hum. Gene Ther., **4**:14-24 (1993)) are employed to transduce mammalian cells with heterologous SMDP and/or SCP nucleic acid. Any of the plasmid expression vectors described herein may be employed in a TfAdpl-DNA complex.

As used herein, "retroviral vector" refers to the well-known gene transfer plasmids that have an expression cassette encoding an heterologous gene residing between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in an appropriate packaging cell line (see, e.g., U.S. Patent 4,650,764).

Suitable retroviral vectors for use herein are described, for example, in U.S. Patent 5,252,479, and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, incorporated herein by reference, which provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, the mouse mammary tumor virus vectors (e.g., Shackleford et al., PNAS, USA, **85**:9655-9659 (1988)), and the like.

In accordance with yet another embodiment of the present invention, there are provided anti-SMDP and/or SCP antibodies having specific reactivity with an SMDP and/or SCP polypeptides of the present invention. Active fragments of antibodies are encompassed within the definition of "antibody". Invention antibodies can be produced by methods known in the art using invention polypeptides, proteins or portions thereof as antigens. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory (1988)), which is incorporated herein by reference. Invention polypeptides can be used as immunogens in generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods

described, for example, in Sambrook et al., supra., and Harlow and Lane, supra. Both anti-peptide and anti-fusion protein antibodies can be used. (see, for example, Bahouth et al., Trends Pharmacol. Sci. **12**:338 (1991); Ausubel et al., Current Protocols in Molecular Biology (John Wiley and Sons, NY (1989) which are incorporated herein by reference).

Antibody so produced can be used, inter alia, in diagnostic methods and systems to detect the level of SMDP and/or SCP present in a mammalian, preferably human, body sample, such as tissue or vascular fluid. Such antibodies can also be used for the immunoaffinity or affinity chromatography purification of the invention SMDP and/or SCP. In addition, methods are contemplated herein for detecting the presence of an invention SMDP and/or SCP protein in a tissue or cell, comprising contacting the cell with an antibody that specifically binds to SMDP and/or SCP polypeptides, under conditions permitting binding of the antibody to the SMDP and/or SCP polypeptides, detecting the presence of the antibody bound to the SMDP and/or SCP polypeptide, and thereby detecting the presence of invention polypeptides. With respect to the detection of such polypeptides, the antibodies can be used for *in vitro* diagnostic or *in vivo* imaging methods.

Immunological procedures useful for *in vitro* detection of target SMDP and/or SCP polypeptides in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by

various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionucleotides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Invention anti-SMDP and/or SCP antibodies are contemplated for use herein to modulate the activity of the SMDP and/or SCP polypeptide in living animals, in humans, or in biological tissues or fluids isolated therefrom. The term "modulate" refers to a compound's ability to increase (e.g., via an agonist) or inhibit (e.g., via an antagonist) the biological activity of an invention SMDP and/or SCP protein, such as the participation in Siah-Mediated-Degradation via an SFC complex and the 26S proteosome. Accordingly, compositions comprising a carrier and an amount of an antibody having specificity for SMDP and/or SCP polypeptides effective to inhibit naturally occurring ligands or other SMDP and/or SCP-binding proteins from binding to invention SMDP and/or SCP polypeptides are contemplated herein. For example, a monoclonal antibody directed to an epitope of an invention SMDP and/or SCP polypeptide including an amino acid sequence set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12 or 14, can be useful for this purpose.

The present invention further provides transgenic non-human mammals that are capable of expressing exogenous nucleic acids encoding SMDP and/or SCP polypeptides. As employed herein, the phrase "exogenous nucleic acid" refers to nucleic acid sequence which is not native to the host, or which is present in the host in other than its native environment (e.g., as part of a genetically engineered DNA construct). In

addition to naturally occurring levels of SMDP and/or SCP, invention SMDPs and/or SCPs can either be overexpressed or underexpressed (such as in the well-known knock-out transgenics) in transgenic mammals.

Also provided are transgenic non-human mammals capable of expressing nucleic acids encoding SMDP and/or SCP polypeptides so mutated as to be incapable of normal activity, i.e., do not express native SMDP and/or SCP. The present invention also provides transgenic non-human mammals having a genome comprising antisense nucleic acids complementary to nucleic acids encoding SMDP and/or SCP polypeptides, placed so as to be transcribed into antisense mRNA complementary to mRNA encoding SMDP and/or SCP polypeptides, which hybridizes to the mRNA and, thereby, reduces the translation thereof. The nucleic acid may additionally comprise an inducible promoter and/or tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of nucleic acids are DNA or cDNA having a coding sequence substantially the same as the coding sequence shown in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. An example of a non-human transgenic mammal is a transgenic mouse. Examples of tissue specificity-determining elements are the metallothionein promoter and the L7 promoter.

Animal model systems which elucidate the physiological and behavioral roles of SMDP and/or SCP polypeptides are also provided, and are produced by creating transgenic animals in which the expression of the SMDP and/or SCP polypeptide is altered using a variety of techniques. Examples of such techniques include the insertion of normal or mutant versions of nucleic acids encoding an SMDP and/or SCP polypeptide by

microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos to produce a transgenic animal. (See, for example, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Laboratory, (1986)).

Also contemplated herein, is the use of homologous recombination of mutant or normal versions of SMDP and/or SCP genes with the native gene locus in transgenic animals, to alter the regulation of expression or the structure of SMDP and/or SCP polypeptides (see, Capecchi et al., Science **244**:1288 (1989); Zimmer et al., Nature **338**:150 (1989); which are incorporated herein by reference). Homologous recombination techniques are well known in the art. Homologous recombination replaces the native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express native (endogenous) protein but can express, for example, a mutated protein which results in altered expression of SMDP and/or SCP polypeptides.

In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both endogenous and exogenous SMDP and/or SCP. Inducible promoters can be linked to the coding region of nucleic acids to provide a means to regulate expression of the transgene. Tissue specific regulatory elements can be linked to the coding region to permit tissue-specific expression of the transgene. Transgenic animal model systems are useful for *in vivo* screening of compounds for identification of specific ligands, i.e., agonists and

antagonists, which activate or inhibit SMDP and/or SCP protein responses.

SMDP and/or SCP proteins, such as Siah-1, are contemplated herein to be a tumor suppressor proteins. Tumor suppressor proteins generally are thought to have a function in signal transduction. Mutation results in loss of function whereupon a signal pathway that the suppressor protein regulates is left in the "on" position, which results in unregulated cell proliferation resulting in cancerous tumor formation. Nearly all tumor suppressors regulate cell division, and proliferation, and may have involvement in biochemical pathways of development and the cell cycle.

The functions of the invention SMDP and/or SCP proteins support the role of both Siah and the invention SMDPs and/or SCPs in cellular pathways that affect protein degradation, such as by activating or inhibiting protein degradation, cell division and proliferation. Accordingly, invention SMDP and/or SCP proteins provide targets for treating a broad variety of pathologies, such as proliferative diseases, cancer pathologies, and the like.

For example, in accordance with yet another embodiment of the present invention, Siah-1 has been found to bind to the protein APC (Kinzler, et al., 1996, supra). The APC protein is known to bind to β-catenin and target it for ubiquitination and degradation (Korinek, V. et al., 1997, Science, 275:1784-1786, Rubinfeld, B. et al., 1997, Science, 275:1790-1792, and Morin, P.J. et al., 1997, Science, 275:1787-1790). Defects in the regulation of the APC/β-catenin pathway for cell growth control have been implicated in a variety

of cancer pathologies, such as epithelial cancers, and the like. Thus, in accordance with the present invention Siah-1, and antagonist or agonists thereof, are contemplated for use in methods for treating a variety of cancers, such as epithelial cancer and the like, preferably by modulating β-catenin degradation. When used for binding to APC, fragments comprising the carboxy terminus of Siah-1, preferably comprising at least amino acids 252-298 of SEQ ID NO:2, are employed (See Figure 3).

Invention nucleic acids, oligonucleotides (including antisense), vectors containing same, transformed host cells, polypeptides and combinations thereof, as well as antibodies of the present invention, can be used to screen compounds *in vitro* to determine whether a compound functions as a potential agonist or antagonist to invention polypeptides. These *in vitro* screening assays provide information regarding the function and activity of invention polypeptides, which can lead to the identification and design of compounds that are capable of specific interaction with one or more types of invention proteins or fragments thereof.

Thus, in accordance with yet another embodiment of the present invention, there are provided methods for identifying compounds which bind to, and preferably, modulate the activity of SMDP and/or SCP polypeptides. The invention proteins may be employed in a competitive binding assay. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to SMDPs and/or SCPs. Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine whether such compounds act as

modulators, agonists or antagonists of invention SMDP and/or SCP proteins. Compounds that bind to and/or modulate invention SMDPs and/or SCPs can be used to treat a variety of pathologies mediated by invention SMDPs and/or SCPs, as described herein.

In accordance with another embodiment of the present invention, transformed host cells that recombinantly express invention polypeptides can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the SMDP-mediated response (e.g., the degradation of a known Siah-mediated target, such as DCC or β-catenin) in the presence and absence of test compound, or by comparing the response of test cells or control cells (i.e., cells that do not express SMDP and/or SCP polypeptides), to the presence of the compound.

As used herein, a compound or a signal that "modulates the activity" of invention polypeptides refers to a compound or a signal that alters the activity of SMDP and/or SCP polypeptides so that the activity of the invention polypeptide is different in the presence of the compound or signal than in the absence of the compound or signal. In particular, such compounds or signals include agonists and antagonists. An agonist encompasses a compound or a signal that activates SMDP and/or SCP protein expression. Alternatively, an antagonist includes a compound or signal that interferes with SMDP and/or SCP expression. Typically, the effect of an antagonist is observed as a blocking of agonist-induced protein activation. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site

specific for agonist binding. A non-competitive antagonist or blocker inactivates the function of the polypeptide by interacting with a site other than the agonist interaction site.

As understood by those of skill in the art, assay methods for identifying compounds that modulate SMDP and/or SCP activity generally require comparison to a control. For example, one type of a "control" is a cell or culture that is treated substantially the same as the test cell or test culture exposed to the compound, with the distinction that the "control" cell or culture is not exposed to the compound. Another type of "control" cell or culture may be a cell or culture that is identical to the transfected cells, with the exception that the "control" cell or culture do not express native proteins. Accordingly, the response of the transfected cell to compound is compared to the response (or lack thereof) of the "control" cell or culture to the same compound under the same reaction conditions.

Accordingly, in accordance with another embodiment of the present invention, there is provided a bioassay for evaluating whether test compounds are capable of acting as agonists or antagonists for SMDP and/or SCP proteins, wherein said bioassay comprises:

(a) culturing cells containing:

DNA which expresses an SMDP and/or SCP or functional fragments thereof,

wherein said culturing is carried out in the presence of at least one compound whose ability to modulate an activity of an SMDP and/or SCP is sought to be

determined, wherein said activity is selected from a protein:protein binding activity or a protein degradation activity and thereafter

(b) monitoring said cells for either an increase or decrease in the level of protein:protein binding or protein degradation.

Methods well-known in the art for measuring protein:protein binding or protein degradation can be employed in bioassays described herein to identify agonists and antagonists of SMDP and/or SCP proteins. For example, the Siah-1 over-expression assay described in Example 14 can be used to evaluate the cell degradation activity of recombinant SMDP and/or SCP proteins or mutants and/or analogs thereof, expressed in mammalian host cells.

As used herein, "ability to modulate protein degradation activity of an SMDP and/or SCP" protein refers to a compound that has the ability to either induce (agonist) or inhibit (antagonist) the protein degradation activity of SMDP and/or SCP proteins within a cell. Host cells contemplated for use in the bioassay(s) of the present invention include human and other mammalian cells (readily avaialable from American Type Culture Collection), as well as genitically engineered yeast or bacteria that express human SMDPs and/or SCPs, and the like.

In yet another embodiment of the present invention, there are provided methods for modulating the protein degradation activity mediated by SMDP and/or SCP protein(s), said method comprising:

contacting an SMDP and/or SCP protein with an effective, modulating amount of an agonist or antagonist identified by the above-described bioassays.

Also provided herein are methods of treating pathologies, said method comprising administering an effective amount of an invention therapeutic composition. Such compositions are typically administered in a physiologically compatible composition.

Exemplary diseases related to abnormal cell proliferation contemplated herein for treatment according to the present invention include cancer pathologies, keratin hyperplasia, neoplasia, keloid, benign prothetic hypertrophy, inflammatory hyperplasia, and the like. Exemplary cancer pathologies contemplated herein for treatment include, gliomas, carcinomas, sarcomas, melanomas, hamartomas, leukemias, lymphomas, and the like.

Also contemplated herein, are therapeutic methods using invention pharmaceutical compositions for the treatment of pathological disorders in which there is too little cell division, such as, for example, bone marrow aplasias, immunodeficiencies due to a decreased number of lymphocytes, and the like. Methods of treating a variety of inflammatory diseases with invention therapeutic compositions are also contemplated herein, such as treatment of sepsis, fibrosis (e.g., scarring), arthritis, graft versus host disease, and the like.

Accordingly, the present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention, such as

pharmaceutical compositions, contain a physiologically compatible carrier together with an invention SMDP and/or SCP (or functional fragment thereof), a SMDP and/or SCP modulating agent, such as a compound (agonist or antagonist) identified by the methods described herein, or an anti-SMDP and/or SCP antibody, as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically compatible" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset, and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well known in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspension, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, as well as

combinations of any two or more thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like, which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid, and the like.

Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium hydroxide, ammonium hydroxide, potassium hydroxide, and the like; and organic bases such as mono-, di-, and tri-alkyl and -aryl amines (e.g., triethylamine, diisopropyl amine, methyl amine, dimethyl amine, and the like) and optionally substituted ethanolamines (e.g., ethanolamine, diethanolamine, and the like).

Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH, physiological saline or both, such as phosphate-buffered

saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary additional liquid phases include glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

As described herein, an "effective amount" is a predetermined amount calculated to achieve the desired therapeutic effect, e.g., to modulate the protein degradation activity of an invention SMDP and/or SCP protein. The required dosage will vary with the particular treatment and with the duration of desired treatment; however, it is anticipated that dosages between about 10 micrograms and about 1 milligram per kilogram of body weight per day will be used for therapeutic treatment. It may be particularly advantageous to administer such compounds in depot or long-lasting form as discussed hereinafter. A therapeutically effective amount is typically an amount of an SMDP and/or SCP-modulating agent or compound identified herein that, when administered in a physiologically acceptable composition, is sufficient to achieve a plasma concentration of from about 0.1 μg/ml to about 100 μg/ml, preferably from about 1.0 μg/ml to about 50 μg/ml, more preferably at least about 2 μg/ml and usually 5 to 10 μg/ml. Therapeutic invention anti-SMDP and/or SCP antibodies can be administered in proportionately appropriate amounts in accordance with known practices in this art.

Also provided are systems using invention SMDPs and/or SCPs, or functional fragments thereof, for targeting any desired protein for ubiquitination and degradation, thus enabling novel gene discovery through functional genomics strategies or providing the basis for ablating target proteins involved in diseases for therapeutic purposes.

In accordance with another embodiment of the invention, there are provided methods for "inducing the degradation of the function" of a desired protein from a particular cell or cell-system. As used herein the phrase "inducing the degradation of the function" refers to deleting, altering, modifying and/or degrading a target protein so that it no longer has the ability to perform its native physiological function. This method is useful to determine the physiological/cellular function of the degraded protein. Thus, this invention method is useful in alleviating one of the rate limiting steps in functional genomics.

The invention methods take advantage of the invention SMDP and/or SCP proteins or other protein-degradation binding domains provided herein to create a system that targets specific proteins for degradation by recruiting them to a SCF complex for ubiquitination and subsequent degradation by an appropriate proteosome, such as 26S proteosome. First, a protein or peptide fragment is selected that binds the protein targeted for degradation in a cell, which is referred to herein as the "target-protein binding domain." Such protein or peptide fragment could be, for example: a domain of any known protein that interacts with the target protein; the Fab region of an anti-target-protein antibody (e.g., sFv, and the like); or a peptide aptamer obtained by screening

(using, for example, yeast two-hybridization, phage display, or other screening methods) a random library of peptide aptamers. The target-protein binding domain is then fused (by engineering cDNAs in expression plasmids to form a chimera) with an appropriate protein-degradation binding domain selected from an invention SMDP and/or SCP, such as Siah, SIP, SAF-1, SAF-2 or SAD, and the like; or other known proteins involved in protein degradation, such as F-box containing proteins, (e.g., Skp1, and the like), SOCS-box containing proteins (see, e.g., Kamura et al., 1998, Genes Dev, **12**:3872; and Starr and Hilton, 1999, Bioessays,**21**:47), HECT family proteins (see, e.g., Huibregtse et al. 1995 Proc. Natl. Acad. Sci. USA **92**:2563-2567), or any other subunit of an E3 ubiquitin ligase complex, and the like (see, e.g., Tyers and Williams, 1999, Science, **284**:601-604; incorporated herein by reference in its entirety).

As used herein, the phrase "protein-degradation binding domain" refers to a protein region that functions to recruit the target protein into a member of the superfamily of E3 ubiquitin ligase complexes, such as the SCF complex, or to Siah-family proteins which may target some proteins for degradation independently of SCF complexes, where the protein-degradation binding domain and/or the target protein become ubiquitinated and then degraded, such as by the 26S proteosome, lysosomes and/or vacuoles (see, e.g., Tyers and Williams, 1999, Science, **284**:601-604; and Ciechanover, 1998, supra). Exemplary protein-degradation domains can be obtained from a protein member of the ubiquitin-mediated protein-degradation family, for example, SIP, Siah, ODC, E7, Fwb7, UB1, Ub4, S5a, antizyme, and others, as disclosed herein and well known to those skilled in the art (see

Table 5). As used herein, the phrase "a protein member of the ubiquitin-mediated protein-degradation family" refers to one of the numerous proteins that are known to interact, via protein:protein binding, in the ubiquitin system of intracellular protein degradation (see, e.g., Ciechanover et al., 1998, supra; and Tyers and Williams, supra, and the like).

In yet another embodiment contemplated by the present invention, methods are provided of identifying a nucleic acid molecules encoding a chimeric protein that modulates a cellular phenotype, said method comprising:

(a) expressing, in a cell, a chimeric nucleic acid comprising a member of a nucleic acid library fused to nucleic acid encoding a protein degradation binding domain of a protein member of the ubiquitin-mediated protein degradation family; and

(b) screening said cells for a modulation of said phenotype.

For example, unbiased nucleic acid libraries can be constructed wherein, each member of the nucleic acid library is expressed as an encoded protein fused to a particular protein-degradation binding domain, such as invention SMDPs and/or SCPs, e.g., Siah-1, SIP (see, e.g., Example 15), SAF-1, SAF-2, or SAD; or other known proteins involved in protein degradation, such as Skp1, F-box containing proteins, HECT family proteins, or any other subunit of an E3 ubiquitin ligase complex, and the like. As used herein a "nucleic acid library" comprises cDNA libraries, YAC libraries, BAC libraries, cosmid libraries, or any other source of nucleic acid encoding polypeptides. The chimeric nucleic acid encoding these

fusion proteins is then introduced into cells possessing a particular phenotype to be assayed.

The cells are then subjected to a "screening" step which comprises selecting one or more cells in which the desired phenotype has been modulated (e.g., suppressed or enhanced). The phenotypes to be screened may be any chemical or physical representation of a cellular process, including but not limited to: cell proliferation in either an attached or detached (i.e., anchorage-independent) state, cell survival, cell death, cell secretion, cell migration, abnormal cell morphology, chemical reactivity (e.g., heavy metals, antibiotics, etc.), physical reactivity (e.g., heat, light, radiation, etc.), and the like.

Next, cDNAs are identified and isolated whose expression products function to modulate the desired phenotype within cells. The cDNAs identified by the invention method encode an invention chimeric protein that interacts, preferably by direct binding, with another protein in the cells that is targeted for degradation, thereby eliminating its physiological function. Accordingly, any target-protein that has one or more protein-binding or protein-interacting partners, or which homodimerizes/homo-oligomerizes is contemplated for degradation in the invention methods. The cDNA identified by the above-described method can be used to perform a two-hybrid screen, as described herein, to identify the protein-binding region of the partner to the target protein, or directly sequenced to determine the identity of the target protein if homo-dimerization or homo-oligomerization situation occurs.

Accordingly, also provided in accordance with the present invention are chimeric proteins, and encoding nucleic acids, comprising a target-protein binding domain operatively linked to a protein-degradation binding domain of a protein member of the ubiquitin-mediated protein-degradation family.

Exemplary proteins whose function can be targeted for degradation according to the invention methods, include any protein encoded by a known gene or cDNA whose function is desired. Exemplary targets include, for example, apoptosis-related proteins, cell-cycle regulatory proteins, heat shock proteins, transcription factors, or any other target protein which, when degraded, will modulate the phenotype of a cell.

Also provided are methods for treating a disease by degrading the function of a target protein, comprising introducing, into a cell, a chimeric protein comprising a target-protein binding domain operatively linked to a protein-degradation binding domain of a protein member of the ubiquitin-mediated protein-degradation family. For example, for a variety of proteins which, when expressed in overabundant or mutated form (e.g., an oncoprotein such as ras, or a genetic mutation, such as in the CF gene (cystic fibrosis gene) result in a known pathology, the chimeric protein of the invention may be used to therapeutically treat the disease, by way of reducing or completely eliminating, via protein degradation, the pathology causing protein. This treatment comprises fusion of a protein domain which binds the target pathology causing protein (i.e., the protein which causes the illness) with a particular protein-degradation binding domain as described herein. This chimeric protein may then be delivered to the

location of the protein which causes the illness by intravenous therapy or gene therapy employing the methods described herein, or any other method well-known to one skilled in the art for delivering a protein to its binding target. As used herein, "treatment of a disease" refers to a reduction in the effects of the disease, including reducing the symptoms of the disease.

In accordance with another embodiment of the present invention, there are provided methods for diagnosing cancer, said method comprising:

detecting, in said subject, a defective sequence or mutant of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13.

In accordance with another embodiment of the present invention, there are provided diagnostic systems, preferably in kit form, comprising at least one invention nucleic acid in a suitable packaging material. The diagnostic nucleic acids are derived from the SMDP and/or SCP-encoding nucleic acids described herein. In one embodiment, for example, the diagnostic nucleic acids are derived from any of SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13. Invention diagnostic systems are useful for assaying for the presence or absence of nucleic acid encoding SMDP and/or SCP in either genomic DNA or in transcribed nucleic acid (such as mRNA or cDNA) encoding SMDP and/or SCP.

A suitable diagnostic system includes at least one invention nucleic acid, preferably two or more invention nucleic acids, as a separately packaged chemical reagent(s) in an amount sufficient for at least one assay. Instructions for use of the packaged reagent are also typically included. Those of skill in the art

can readily incorporate invention nucleic probes and/or primers into kit form in combination with appropriate buffers and solutions for the practice of the invention methods as described herein.

As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as invention nucleic acid probes or primers, and the like. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. The packaging material has a label which indicates that the invention nucleic acids can be used for detecting a particular sequence encoding SMDP and/or SCP including the nucleotide sequences set forth in SEQ ID NOs:1, 3, 5, 7, 9, 11 and 13 or mutations or deletions therein, thereby diagnosing the presence of, or a predisposition for, cancer. In addition, the packaging material contains instructions indicating how the materials within the kit are employed both to detect a particular sequence and diagnose the presence of, or a predisposition for, cancer.

The packaging materials employed herein in relation to diagnostic systems are those customarily utilized in nucleic acid-based diagnostic systems. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits an isolated nucleic acid, oligonucleotide, or primer of the present invention. Thus, for example, a package can be a glass vial used to contain milligram quantities of a contemplated nucleic acid, oligonucleotide or primer, or it can be a microtiter plate well to which microgram

quantities of a contemplated nucleic acid probe have been operatively affixed.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

All U.S. patents and all publications mentioned herein are incorporated in their entirety by reference thereto. The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1982); Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol. **152**, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987)).

Two-hybrid assays.

Library screening by the yeast two-hybrid method was performed herein as described (Durfee et al., 1993; Sato et al., 1995; Matsuzawa et al. 1998) using the pGilda plasmid encoding the desired amino acid region as bait, an appropriate cDNA library, and the EGY48 strain S.cerevisiae (MATa, trp1, ura3, his, leu2::plexApo6-leu2). Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino-acids as described previously (Sato et al., 1994). Transformations were performed by a LiCl method using 0.25 mg of pJG4-5-cDNA library DNA, and 5 mg of denatured salmon sperm carrier DNA. Clones that formed on Leu deficient BMM plates containing 2% galactose/ 1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for β-galactosidase measurements as previously described.

1. Yeast two-hybrid screen of BAG-1 binding proteins to obtain cDNA encoding Siah-1α.

The mouse BAG-1 amino acid sequence was cloned into the pGilda plasmid and used as bait to screen a human Jurkat T-cell cDNA library. From an initial screen of ~1.6 X 10^7 transformants, 298 clones were identified that trans-activated the LEU2 reporter gene based on ability to grow on leucine-deficient media. Of those, 30 colonies were also positive for β-galactosidase. These 30 candidate transformants were then cured of the LexA/BAG-1 bait plasmid by growth in media containing histidine and then mated with each of 5 different indicator strains of cells containing one of following

LexA bait proteins: BAG-1 (1-219), Bax (1-171), v-Ras, Fas (191-335), or Lamin-C. The mating strain was RFY206 (MATa, his3D200, leu2-3, lys2D201, ura3-52, trp1D::hisG), which had been transformed with pGilda-BAG-1 or various control proteins and selected on histidine-deficient media. This resulted in 23 clones which displayed specific two-hybrid binding interactions with BAG-1. DNA sequencing analysis revealed 4 cDNAs encoding portions of Siah-1.

2. Isolation of full-length human Siah-1α cDNAs.

To obtain the complete sequence of human Siah-1, cDNA fragments containing the 5' end of human Siah 1 were PCR-amplified from Jurkat randomly primer cDNAs by using aforward primer 5' GGGAATTCGGACTTATGGCATGTAAACA-3' (SEQ ID NO:42) containing an EcoRI site and a reverse primer 5' TAGCCAAGTTGCGAATGGA-3' (SEQ ID NO:43), based on sequences of EST database clones (NCBI ID: AA054272, AA258606, AA923663, AA418482, and AI167464). The PCR products were digested with EcoRI and BamHI, then directly subcloned into the EcoRI and SalI sites of pcI plasmid into which the cDNA derived from pJG4-5-Siah (22-298) had previously been cloned, as a BamHI - XhoI fragment. The complete human Siah-1α cDNA and amino acid sequence is set forth in SEQ ID Nos:1 and 2, respectively. The human Siah-1α sequence contains 16 N-terminal amino acids that are not present in the human Siah-1β protein.

3. Yeast two-hybrid screen of Siah-1 binding proteins to obtain cDNA encoding SIP-L and SIP-S.

Human Siah-1α cDNA encoding amino acids 22-298 of SEQ ID NO:1 (corresponding to amino acids 6-282 set

forth in Nemani et al., supra) was cloned into the pGilda plasmid and used as a bait to screen a human embryonic brain cDNA library (Invitrogen) in EGY48 strain S.cerevisiae. From an initial screen of ~2.0 X 10^7 transformants, 322 clones were identified that trans-activated the LEU2 reporter gene based on ability to grow on leucine-deficient media. Of those, 32 colonies were also positive for β-galactosidase. These 32 candidate transformants were then cured of the LexA/Siah-1 bait plasmid by growth in media containing histidine and then mated with each of 5 different indicator strains of cells containing one of following LexA bait proteins: Siah-1(22-298), Bax (1-171), v-Ras, Fas (191-335), or BAG-1. The mating strain was RFY206 which had been transformed with pGilda-Siah-1 or various control proteins and selected on histidine-deficient media. This resulted in 11 clones which displayed specific two-hybrid interactions with Siah-1. DNA sequencing analysis revealed 5 cDNAs encoding portions of SIP-L, 1 cDNA encoding portions of SIP-S, 3 cDNAs encoding portions of of APC(2681-2843), and 2 cDNAs encoding portions of Siah-1. The SIP-L and SIP-S clones were sequenced and the resulting nucleotide sequences are set forth in SEQ ID Nos:3 and 5, respectively.

4. Yeast two-hybrid screen of Skp1 binding proteins to obtain cDNA encoding SAF-1 and SAD.

Human Skp1 cDNA encoding amino acids 91-163 of (Zhang et al., 1995, Cell, **82**:915-925) was cloned into the pGilda plasmid as a bait to screen a human embryonic brain cDNA library (Invtrogen) in EGY48 strain S.cerevisiae. From an initial screen of ~1.2 X 10^8 transformants, 130 clones were identified that trans-activated the LEU2 reporter gene based on ability to grow

on leucine-deficient media. Of those, 36 colonies were also positive for β-galactosidase. These 36 candidate transformants were then cured of the LexA/BAG-1 bait plasmid by growth in media containing histidine and then mated with each of 5 different indicator strains of cells containing one of following LexA bait proteins: Skp1 (91-163), SIP-L, Bax (1-171), v-Ras, Fas (191-335), or Siah-1. The mating strain was RFY206 which had been transformed with pGilda-Skp1 or various control proteins and selected on histidine-deficient media. This resulted in 3 clones which displayed specific two-hybrid interactions with Skp1 and 18 clones clones which displayed specific two-hybrid interactions with both Skp1 and SIP-L. DNA sequencing analysis revealed 12 cDNAs encoding portions of SAF-1 and 9 cDNAs encoding portions of SAD. The SAF-1 and SAD clones were sequenced and the resulting nucleotide sequences are set forth in SEQ ID Nos:7 (SAF-1α), 9 (SAF-1β), and 13 (SAD).

5. Isolation of full-length SAF-2 cDNAs.

Full-length cDNA encoding a human SAF-2 protein was PCR-amplified from ZAPII Jurkat cDNA labrary (Stratagene) by using a forward primer 5'-GTGAATTCATGCAACTTGTACCTGATATAGAGTTC-3' (SEQ ID NO:44) containing an EcoRI site and a reverse primer 5'-GGACTCGAGGCTCTACAGAGGCC-3' (SEQ ID NO:45), based on human DNA sequence from clone 341E18 on chromosome 6p11.2-12.3 (AL031178). The PCR products were digested with EcoRI and XhoI, then directly subcloned into the EcoRI and XhoI sites of the plasmid pCDNA3. The corresponding plasmid was sequenced and the results are set forth in SEQ ID Nos: 11 and 12.

6. Yeast two-hybrid screen of SIP-L binding proteins.

The human SIP-L cDNA encoding full-length SIP-L was cloned into the pGilda plasmid as a bait to screen a human embryonic brain cDNA library (Invtrogen) in EGY48 strain S.cerevisiae. From an initial screen of ~1.5 X 10^7 transformants, 410 clones were identified that trans-activated the LEU2 reporter gene based on ability to grow on leucine-deficient media. Of those, 68 colonies were also positive for β-galactosidase. These 32 candidate transformants were then cured of the LexA/SIP-L bait plasmid by growth in media containing histidine and then mated with each of 32 different indicator strains of cells containing one of following LexA bait proteins: SIP-L, Bax (1-171), v-Ras, Fas (191-335), or BAG-1. The mating strain was RFY206 which had been transformed with pGilda-SIP-L or various control proteins and selected on histidine-deficient media. This resulted in 16 clones which displayed specific two-hybrid interactions with SIP-L. DNA sequencing analysis revealed 3 cDNAs encoding portions of Skp1, 1 cDNA encoding portions of Siah-1, and 11 cDNAs encoding portions of SIP-L. These results indicate that SIP-L binds to Skp1 and Siah-1 proteins, and is able to homodimerize with SIP isoforms.

7. A cell proliferation functional assay of SIP/Siah interaction

The effects of invention SIP-L and SIP-S proteins on Siah-1-induced cell cycle arrest in 293T epithelial cancer cells was examined and the results are shown in Figure 4. Human embryonic kidney 293 cells were maintained in high-glucose DMEM medium containing 10% fetal calf serum, 1 mM L-glutamine, and antibiotics. Cells (~5 x 10^5) in 60 mm plates were transfected with a

total of 3.0 µg of plasmid DNAs encoding Siah-1 alone or together with SIP or SIP-S by a calcium phosphate precipitation technique. After 24 hours, the cells were harvested and the number of viable and dead cells were counted using trypan blue dye exclusion assays. Efficiency of transient transfection was estimated by in situ β-galactosidase assay using a portion of the transfected cells. The transient transfection efficiency of the T293 cells was consistently 90%.

As revealed in Figure 4, over-expression of Siah-1 resulted in decreased numbers of viable cells after 24 hours, without an increase in cell death. Thus, Siah-1 suppresses proliferation of 293 cells. Co-transfection of SIP-L with Siah-1 did not substantially alter Siah-1-mediated growth suppression. In contrast, the SIP-S protein abrogated the growth suppressive effects of Siah-1, which indicates that the invention SIP-S protein affects Siah-1 intracellularly in a different manner than SIP-L.

8. In vitro SIP:Siah-1 protein interaction assays.

Complementary cDNA encoding SIP-L was cloned into pGEX-4T-1 and expressed in XL-1-blue cells (Stratagene, Inc.), and affinity-purified using glutathione-Sepharose as is well-known in the art. Purified GST-fusion proteins (0.5-1.0 µg immobilized on 10-20 µl of glutathione beads) and 2.5 ul of rat reticulocyte lysates (TNT-Lysates; Promega, Inc.) containing 35S-labeled in vitro translated (IVT) Siah-1 proteins were incubated in 0.1 ml of HKMEN (10 mM HEPES [pH7.2], 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.1% NP-40) at 4°C for 30 minutes. The beads were washed 3X with 1 ml HKMEN solution, followed by boiling in 25 µl of Laemmli-

SDS sample buffer. The eluted proteins were analyzed by SDS-PAGE (12%) and detected by fluorography. Use of equivalent amounts of intact GST-fusion proteins and successful IVT of each protein was confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively.

The results are shown in Figure 5A and indicate that Siah-1 binds to SIP-L and homodimerizes in vitro.

9. Co-immunoprecipitation Assay of SIP:Siah-1.

Two x 10^6 293T cells in 100 mm plates were transiently transfected with 10 µg of pCDNA3-myc-SIP-L and 10 mg of pcDNA3-HA-Siah-1 (amino acids 97-298 of SEQ ID NO:2). Twenty-four hours later, cells were disrupted by sonication in 1 ml of HKMEN solution containing 0.2% NP-40, 0.1 µM PMSF, 5 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin. After preclearing with normal mouse IgG and 10 ml protein A-agarose, immunoprecipitations were performed using 10 ml of anti-myc antibody-conjugated sepharose (Santa Cruz) to precipitate the myc-SIP-L fusion, or an anti-IgG as a control at 4°C for 4 hours. After extensive washing in HKMEN solution, immune-complexes were analyzed by SDS-PAGE/immunoblotting using anti-HA antibody 12CA5 (Boehringer Mannheim), followed by HRPase-conjugated goat anti mouse immunoglobulin (Amersham, Inc.), and detected using an enhanced chemiluminescence (ECL) system (Amersham, Inc.).

The results are shown in Figure 5B and indicate that SIP proteins bind to Siah-1 intracellularly.

10. Yeast two-hybrid assay of Siah-1:APC binding specificity.

One µg of plasmids encoding fusion proteins of the LexA DNA-binding domain fused to Siah-1, APC(2681-284), BAG-1, Bax, Ras, Fas, FLICE were co-tansformed into yeast strain EGY48 with 1 µg of pJG4-5 plasmid encoding fusion proteins of the B42 trans-activation domain fused to APC(2681-2843) and Siah-1. Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control which resulted in equivalent amounts of growth for all transformants. Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-galactosidase activity of each colony was tested by filter assay and scored as blue (+) versus white (-) after 60 minutes.

The results are shown in Table 1, and indicate that APC interacts specifically by direct binding with Siah-1, and not with BAG-1, Bax, Ras, Fas nor FLICE.

Table 1: **Specific Interaction of Siah with SIP**

Lex A	**B42**	**Leu$^+$**	**β-Gal$^+$**
Siah-1	APC (2681-2843)	+	+
APC (2681-2843)	Siah-1	+	+
BAG-1	APC (2681-2843)	-	-
Bax	APC (2681-2843)	-	-
Ras	APC (2681-2843)	-	-
Fas	APC (2681-2843)	-	-
FLICE	APC (2681-2843)	-	-
empty	APC (2681-2843)	-	-

11. Yeast two-hybrid assay of Siah-1:SIP binding specificity.

One μg of plasmids encoding fusion proteins of the LexA DNA-binding domain fused to Siah-1, Siah-2, BAG-1, Bax, Ras, Fas, FLICE, and SIP-L were co-tansformed into yeast strain EGY48 with 1 μg of pJG4-5 plasmid encoding fusion proteins of the B42 trans-activation domain fused to SIP-L, SIP-S, Siah-1, Siah-2, BAG-1, Bax, and Ras. Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control which resulted in equivalent amounts of growth for all transformants. Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-galactosidase activity of each colony was tested by filter assay and scored as blue (+) versus white (-) after 60 minutes.

The results are shown in Table 2, and indicate that SIP proteins interact specifically by direct binding with Siah proteins. SIP-L was found to interact with Siah-1 and Siah-2, and not with BAG-1, Bax, Ras, Fas nor FLICE. SIP-S was also found to interact with Siah-1. Table 2 also reveals that the SIP-L homodimerization domain is within amino acids 73-228 of SIP-L (SEQ ID NO:4)

Specific Interaction of Siah with SIP

Table 2

Lex A	**B42**	**Leu$^+$**	**β-Gal$^+$**
Siah-1	SIP-L	+	+
Siah-1	SIP-S	+	+
Siah-2	SIP-L	+	+
BAG-1	SIP-L	-	-
Bax	SIP-L	-	-
Ras	SIP-L	-	-
FLICE	SIP-L	-	-
empty	SIP-L	-	-
SIP-L	Siah-1	+	+
SIP-L	Siah-2	+	+
SIP-L	BAG-1	-	-
SIP-L	Bax	-	-
SIP-L	Ras	-	-
SIP-L	SIP-L	+	+
SIP-L	SIP-S	-	-

12. Mapping of Siah-APC interaction domains.

Expression plasmids encoding fusion proteins of Siah-1α fragments corresponding to: SEQ ID NO:2 amino

acids 22-298; 22-251; 22-193; 97-298; and 46-102, fused to the B-42 trans-activation domain were co-transformed into yeast EGY48 cells with a plasmid encoding a chimeric fusion protein of the Lex A DNA-binding domain fused to amino acids 2681-2843 of APC "APC(2681-2843)." Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control. Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-galactosidase activity for each colony was tested by filter assay and scored as blue (+) versus white (-) (β-gal) based on a 1 hour of color development.

The results are shown in Figure 3 and indicate that a region within the 47 carboxy terminal amino acids of Siah-1α (SEQ ID NO:2) is required for binding to APC.

13. Mapping of SKP-1, SIP-L, SAF-1, and SAD interaction domains.

Expression plasmids encoding fusion proteins of SAF-1α and functional fragments thereof corresponding to SEQ ID NO:8 amino acids 68-443; 80-443; and 258-443, were fused to the B-42 trans-activation domain. Likewise, expression plasmids encoding fusion proteins of SAD and functional fragments thereof corresponding to SEQ ID NO:14 amino acids 128-447; and 360-447, were fused to the B-42 trans-activation domain. These SAF-1-fragment- and SAD-fragment-B-42 fusion proteins were co-transformed into yeast EGY48 cells with a plasmid encoding a chimeric fusion protein of the Lex A DNA-binding domain fused to either SKP1, SIP-L, SAF-1, or SAD. Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control. Plasmid combinations that resulted in growth on leucine-deficient media within

4 days were scored as positive (+). β-galactosidase activity for each colony was tested by filter assay and scored as blue (+) versus white (-) (β-gal) based on a 1 hour of color development.

The results are shown in Figure 6A and 6B. Figure 6A indicates that SAF-1 interacts by direct binding to Skp1, SIP-L and SAD, but does not interact with Siah-1. A region within the SAF-1 fragment corresponding to amino acids 80-257 of SEQ ID NO:8 is required for SIP-L interaction, whereas a region within amino acids 258-443 of SAF-1 is required for Skp1 and SAD interaction.

Figure 6B indicates that SAD interacts by direct binding to Skp1, SIP-L and SAF-1, but does not interact with Siah-1. A region within the SAD fragment corresponding to amino acids 1-127 of SEQ ID NO:14 is required for SAF-1 interaction; a region within amino acids 128-359 of SAD is required for Skp1 interaction; and a region within amino acids 360-447 of SEQ ID NO:14 is required for SIP-L interaction.

14. Effect of Siah-1 over-expression on stability of β-catenin.

293T cells were transiently transfected with a plasmid encoding myc-tagged β-catenin and either pcDNA3, pcDNA3-Siah-1, or pcDNA3-Siah-1(97-298; amino acids 97-298 of SEQ ID NO:2). Whole cell lysates were prepared, normalized for total protein content (25 µg per lane) and analyzed by SDS-PAGE/immunoblotting using an anti-Myc tag antibody.

Figure 7 indicates that expression of full-length Siah-1 abolishes, by degradation, the presence of β-catenin within cells, whereas expression of amino acids 97-298 of Siah-1 (SEQ ID NO:2) does not result in β-catenin degradation. Thus, a region within amino acids 1-96 of SEQ ID NO:2 (Siah-1α), which contains the N-terminal "Ring" domain, is required for protein degradation.

15. Demonstration of SIP-mediated degradation of a target protein, TRAF6.

An invention SIP-based method for targeted degradation of proteins was applied to the degradation TRAF proteins. The schematic in Figure 9 shows the strategy employed for targeted degradation of specific TRAF-family proteins. A chimeric protein is expressed from the plasmid pcDNA3 in which SIP-L (SEQ ID NO:3) is fused with bacterial thioredoxin containing various TRAF-binding peptides displayed on the surface of thioredoxin, as described by Brent and colleagues (Colas, et al. Nature, **380:** 548, 1996; Cohen, et al. Proc. Natl. Acad. Sci., **95:** 14272, 1998; Geyer, et al. Proc. Natl. Acad. Sci., **96:** 8562, 1999; Fabbrizio, et al. Oncogene, **18:** 4357, 1999). The TRAF-binding peptide binds to a member of the TRAF-family, and targets the TRAF-protein for ubiquitination and subsequent proteosome-dependent degradation because the SIP-region of the chimeric protein recruits ubiquitin-conjugating enzymes (E2s) to the protein complex.

Isolation of target-protein binding domain peptides that selectively bind TRAF2 and TRAF6.

A peptide aptamer library was screened by the yeast two-hybrid method to identify peptides that bind to either TRAF2 or TRAF6 using the methods described in Leo, et al. J Biol Chem, **274:**22414, 1999. TRAFs are a family of signal transducing proteins involved in cytokine receptor signaling inside cells. The sequences of the resulting TRAF-binding peptides are set forth in (Tables 3 and 4).

TABLE 3

Selected Traf 2 Aptamer Clones

Clones	(SEQ ID NO:)	SLxCIxLR motif
219	(15)	SESPGALRSGSLRCISLRIC
230	(16)	VCRGRIRSGSLRCISLRICR
221	(17)	LLRLGCIRLLMLRRGVVFRL
208	(18)	VLFLSLRFWGLNIVVMGRLL
215	(19)	CRSLGVIVGGTEAAGAPTFI
		LS motif
208	(20)	VLFLSLRFWGLNIVVMGRLL
213	(21)	WLRRGLVGVFFLLSRVMVGI
218	(22)	SLGLSVCIGRRAGGGFRGFG
237	(23)	RFALSIGVCVVVRVGICLGM
		LV motif
209	(24)	SAVLVLVYVSAALRGRGFGI
227	(25)	HGGGRGALVSVMYLCGFIRL
		Non-Consensus motif
231	(26)	RGRVIGMWVGLRCRMFLV

TABLE 4

Selected Traf 6 Aptamer Clones

Clones	(SEQ ID NO:)	WR motif
625	(27)	VDWAVYSVVWRYTTT*
631	(28)	KTSVILVWRLSLFFCLYRSL*
606	(29)	ANRCWRE*

	628	(30)	EGTLSKRMWRTHN*
	640	(31)	SWRDMTQSGM*
	604	(32)	DVPWQRACARQ*
	607	(33)	LERVARWVL*
5	602	(34)	VADVLVFWGYVF*
			DVxVF motif
	602	(34)	VADVLVFWGYVF*
	613	(35)	GDVGVFPE*
			Non-Consensus motif
10	603	(36)	PEMMLEGPKYCLxLxE*
	609	(37)	LLYGALA*
	612	(38)	GAIKFAHESCE*
	616	(39)	PMAMD*
	632	(40)	QEEEM*
15	639	(41)	ISVVHGIGSDSD*

* Termination codon

SIP-fusion Chimeric protein construction:

An invention SIP-fusion chimeric construct is generated by combining the open reading frame (ORF) of SIP_L, followed immediately by restriction enzyme sites allowing for subcloning of desired target-protein-binding domains (e.g. peptides or protein domains). These SIP-fusions are then transfected into mammalian cells to eliminate by protein degradation specific target proteins which bind the subcloned peptides/protein domains by recruiting them into the ubiquitin conjugating complex.

The parent SIP-vector (SIPpcDNA3.1) cassette was engineered as follows:

Oligonucleotides corresponding to the 5' and 3'end of SIP_L were used in PCR to amplify the entire ORF of SIP_L (SEQ ID NO:3). The forward primer contains a *Hind III* restriction site linker (5'-GATCAAGCTTATGGCTTCAGAAGAGCTACAG; (SEQ ID NO:46) restriction site is underlined) followed immediately by

the SIP_L (SEQ ID NO:3) start codon; the reverse primer contains an *EcoRI* restriction site and mutations in the stop codon allowing for translational readthrough (5'-GATCGAATTCtccAAATTCCGTGTCTCCTTTGGCTTG; (SEQ ID NO:47) mutated stop codon is in lowercase). The generated PCR product was then agarose gel-purified and digested with *Hind III* and *EcoRI* restriction enzymes (New England BioLabs; Beverly, MA). The product was again gel-purified before ligating into *Hind III/EcoRI* digested pcDNA3.1 expression vector (Invitrogen; Carlsbad, CA) with T4-DNA ligase (New England BioLabs). This construct was termed SIPpcDNA3.1.

For the construction of SIP-thioredoxin (Trx) peptide-aptamer fusions, clones from a peptide-aptamer library screened against Traf6 (see Table 4) were amplified by PCR with the following primers: Forward: 5'-CCTCTGAATTCCATATGAGCGATAAAATTATTCACC (SEQ ID NO:48) *EcoRI* underlined; Reverse: 5'-GATCCTCGAGTAGATGGCCAGCTAGGCCAGGTTA (SEQ ID NO:49) *Xho I* underlined.

The resulting PCR products (~350-370bp) contain the ORF of thioredoxin (Trx) with the selected peptide aptamers inserted into its active-loop. The products were then digested with *EcoRI* and *Xho I* before ligating into the *EcoRI/XhoI*-digested SIPpcDNA3.1 cassette using T4-DNA ligase. Final clone constructs were numbered and were confirmed by sequencing before using in transfection studies.

Tranfection:

HEK293T cells were transiently transfected by a lipofectamine method with various amounts (1 vs 4 μg) of pcDNA3 plasmids encoding either SIP-TR fusion protein lacking a TRAF6-binding peptide ("SIP") or SIP-TR fusion protein displaying one of the peptides shown in Table 4 above (set forth in Figure 10 as S603, S604, S606). In some cases, the proteosome inhbitor MG132 (10 μM) was added to cultures to prevent protein turnover. SIP* in Figure 10 corresponds to the control expression product of parental construct SIP pcDNA3.1

To determine the efficacy of the SIP:TRAF-binding peptide chimeric proteins, levels of TRAF6 protein were then measured two days later by immunoblotting using a anti-TRAF6-specific antiserum (Santa Cruz Biotech, Inc.) in experiments where HEK293T cell lysates were normalized for total protein content (25 μg per lane). The cell lysates were analyzed by SDS-PAGE/immunoblotting using an enhanced chemiluminescence detection method, as described previously (Leo, et al. J Biol Chem, **274:** 22414, 1999). The results shown in the left panel of Figure 10 show that SIP-TR fusion proteins displaying TRAF6-binding peptides (S603, S604, and S613) induce a reduction in TRAF6 protein levels, with the S603 peptide representing the most potent of these.

To determine the specificity of the SIP:TRAF-binding peptide chimeric proteins, the same immunoblots were reprobed with an antiserum against SIP to demonstrate equivalent levels of production of SIP-TR fusion proteins, or with antibodies specific for TRAF2 to reveal selective degradation of TRAF6 but not TRAF2. The results shown in the right panel of Figure 10 show that

addition of a proteosome inhibitor, MG132, prevents the reductions in TRAF6. Note also that TRAF2 protein is not degraded, demonstrating the specificity of the targeting approach.

16. Demonstration of Ornithine decarboxylase-(ODC) mediated degradation of a target protein.

The levels of intracellular proteins are regulated by proteasome-dependent proteolysis. The selective degradation of cellular proteins is mediated primarily by both the ubiquitin-dependent and -independent proteasome pathways. In this example, the substrate receptor of a major proteolytic machinery is engineered to direct the degradation of otherwise stable cellular proteins in mammalian cells.

Strategy for the selective degradation of cellular protein by chimeric ODC proteins and antizyme.

Ornithine decarboxylase (ODC) is degraded in a 26S proteasome antizyme-dependent manner, which does not require ubiquitination. In order to target the protein of interest (Targets), the adapter protein or peptide, which binds to target protein, is covalently fused to ODC for the degradation of protein complex by the 26S proteasome. A model of antizyme-dependent targeted protein degradation by ODC-conjugated proteins is shown in Figure 11A. Figure 11B shows the amino acid sequence of the ODC-fusion protein (SEQ ID NO:50)(human ODC sequence GenBank M16650).

Targeted degradation of TRAF6 by ODC-TRAF6C and ODC-RANK peptide.

For targeted degradation of TRAF6, HEK293T cells were transiently transfected with plasmid encoding HA-TRAF6 (0.5 μg), HA-TRAF2 (0.5 μg), ODC (0.5 μg), ODC-TRAF6C (0.5 μg), ODC-RANKpeptide (0.5 μg) ODC-CD40CT (0.5 μg) or myc-Antizyme (0.5 μg) in various combinations, as indicated in Figure 12 (total DNA amount normalized). After 24 h, cell lysates were prepared from duplicate dishes of transfectants, normalized for total protein content (20 μg per lane), and analyzed by SDS-PAGE. Immunoblotting was performed using antibodies specific for the tags HA (TRAF6) or Myc (ODC or Antizyme), with detection by enhanced chemiluminescence (ECL). Levels of TRAF6 mRNA were measured by Northern blot (mRNA).

Expression of ODC-TRAF6C in HEK293T cells induced marked reductions in HA-TRAF proteins, with or without co-expressing plasmids encoding antizyme. In contrast, expression of ODC-TRAF6-RANK peptide decreased TRAF6 proteins in an antizyme-dependent manner. Expression of ODC-CD40 did not reduce TRAF6 protein. Neither ODC-TRAF6C nor ODC-RANK peptide affected the expression of TRAF2 proteins. Moreover, reductions in TRAF6 protein levels were not due to a decrease in TRAF6 mRNA, as determined by northern blot. Therefore, the degradation of TRAF6 by ODC-TRAF6C was antizyme-independent but the degradation of TRAF6 by ODC-RANK peptide was antizyme-dependent.

Other examples for ODC-adapter-induced degradation of target protein(s).

The activity of ODC-adapter-induced degradation of other target protein(s) was tested. Antizyme-dependent targeted degradation of retinoblastoma (Rb) by ODC-E7 peptide is shown in Figure 13A. HEK293T cells were transiently transfected with plasmid encoding HA-Rb (0.5 μg), ODC (0.5 μg), ODC-E7 peptide (0.5 μg) or myc-Antizyme (0.5 μg) in various combinations, as indicated in Figure 13A (total DNA amount normalized). Antizyme-independent targeted degradation of Cdk2 by ODC-p21waf-1 is shown in Figure 13B. HEK293T cells were transiently transfected with plasmid encoding myc-Cdk2 (0.5 μg), ODC (0.5 μg), ODC-p21waf-1 (0.5 μg) or myc-Antizyme(0.5 μg) in various combinations, as indicated in Figure 13B (total DNA amount normalized). Antizyme-independent targeted degradation of IKKβ by ODC-IKKβ (leucine-zipper domain) is shown in Figure 13C. HEK293T cells were transiently transfected with plasmid encoding HA-IKKβ (0.5 μg), ODC (0.5 μg), ODC-IKKβ-LZ(0.5 μg) or myc-Antizyme (0.5 μg) in various combinations, as indicated in Figure 13C (total DNA amount normalized). After 24 h, cell lysates were prepared from duplicate dishes of transfectants, normalized for total protein content (20 μg per lane), and analyzed by SDS-PAGE. Immunoblotting was performed using antibodies specific for Rb, Cdk2, IKKβ or HSC70 (as a control), with ECL-based detection.

Analysis of interactions of ODC-TRAF6C and TRAF6.

For analysis of interactions of ODC-TRAF6C and TRAF6, HEK293T cells were transiently transfected with plasmids encoding hemaglutinin (HA)-tagged TRAF6 and ODC

(0.5 µg), ODC-TRAF6C peptide (0.5 µg) or Antizyme (0.5 µg) in various combinations, as indicated in Figure 14 (total DNA amount normalized). After 24 h, 10 µM MG132 was added into culture media, and the cells were incubated another 6 hours. Lysates were normalized for total protein content and subjected to immunoprecipitation using 20 µl of anti-myc monoclonal antibody-conjugated beads. After recovering immune-complexes with beads and washing, the immunoprecipitates were analyzed by SDS-PAGE. Immunoblotting was performed using an anti-HA monoclonal antibody, with ECL-based detection. As a control, 0.1 volume of input cell lysate was loaded directly in the same gel.

To preliminarily assess whether ODC-TRAF6 and antizyme can exist in a complex with TRAF6, the TRAF6 proteins were tested for co-immunoprecipitation with ODC-TRAF6C using monoclonal antibodies against the myc epitope tag. As shown in Figure 14, TRAF6 was recovered in TRAF6C-ODC, but not control ODC-empty, immune-complexes prepared from cells expressing ODC-TRAF6C.

<u>Targeted degradation of TRAF6 by ODC-RANK peptide is proteasome-dependent.</u>

To test whether targeted degradation of TRAF6 by ODC-RANK peptide is proteasome-dependent, HEK293T cells were transiently transfected with 0.2 µg plasmid encoding HA-TRAF6 (0.5 µg), ODC-RANK peptide (0.5 µg) or myc-Antizyme (0.5 µg) in various combinations, as indicated in Figure 15 (total DNA amount normalized). After 24 h, cells were either untreated or treated with 1 µM MG132 (MG132), 1 nM Epoximycine (Epox.), 10 µM

Lactastacine (Lact.) or 1 μM Trypsin inhibitor (Tryp.) for 6 hours. Cell lysates were prepared from duplicate dishes of transfectants, normalized for total protein content (20 μg per lane), and analyzed by SDS-PAGE. Immunoblotting was performed using antibodies specific for HA.

As shown in Figure 15, degradation of TRAF6 by ODC-RANK peptide and antizyme was inhibited by proteasome inhibitors, MG132, Epoximycine and Lactastacine but not by trypsin inhibitor. These results indicate that the degradation is S26 proteasome dependent.

Pulse-chase analysis of TRAF6 turnover rate.

Pulse-chase analysis of ectopically expressed HA-tagged TRAF6 was performed. HEK293T cells were transiently co-transfected with plasmids encoding HA-TRAF6 and ODC-RANK peptide, with or without myc-Antizyme. After 24 hours, cells were pulse-labeled with ^{35}S-methionine and cysteine, and then chased with media lacking the labeled amino acids. Cells were lysed at the indicated times (Figure 16), and the expressed HA-TRAF6 was recovered by immunoprecipitation via a HA epitope tag. Immunoprecipitad HA-TRAF6 was subjected to SDS-PAGE, and dried gels were analyzed with a PhosphorImager. Data from pulse-chase analysis is presented as the average ± SD from duplicate experiments (Figure 16B; -antizyme, closed circles; +antizyme, open circles). The blots shown are representative of duplicate experiments (Figure 16A).

The turnover rate of the TRAF6 was increased in the 293T cells transiently transfected with ODC-RANK peptide and antizyme, as compared to the cells tranfected with

ODC-RANK peptide alone. These results demonstrate that ODC-RANK peptide and antizyme promote down-regulation of TRAF6 in a post-translational manner.

Functional analysis using ODC-E7 peptide.

To assess whether ODC-E7 peptide reduces endogenous Rb protein level and affects its cellular function, HEK293T cells were transiently transfected with ODC-E7 peptide and antizyme. Degradation of endogenous Rb protein by ODC-E7 is shown in Figure 17A. HEK293T cells (100 mm dish) were transiently transfected with 2 μg plasmid encoding ODC (2 μg), ODC-E7 peptide (2 μg) or myc-Antizyme (2 μg) in various combinations, as indicated in Figure 17A (total DNA amount normalized). After 48 h, lysates were normalized for total protein content and subjected to immunoprecipitation using 1 μg of anti-Rb monoclonal antibody. After recovering immune-complexes with protein G and washing, the immunoprecipitates were analyzed by SDS-PAGE. Immunoblotting was performed using an anti-Rb monoclonal antibody with ECL-based detection.

The effect of ODC-E7 on E2F reporter activity was also tested. HEK293T cells were transiently transfected with a reporter gene plasmid (0.1 μg) that contains a E2F responsive element cloned upstream of a luciferase reporter gene, together with 0.01 μg of pCMVβ-gal as a transfection-efficiency control, and 0.1 μg of the indicated plasmids encoding ODC, ODC-E7 or Antizyme in various combinations, as indicated in Figure 17B (bars correspond to plasmid combinations as indicated in Figure 17A)(total DNA amount normalized). Luciferase activity was measured in cell lysates 24 hr later and normalized relative to β-galactosidase (mean ± std. dev.; n = 3).

As shown in Figure 17A, endogenous Rb protein was degraded by ODC-E7 peptide with antizyme. Since transcription factor E2F activity is normally suppressed by Rb in proliferating cells, the effects of ODC-E7 and antizyme on E2F activity was explored using transient transfection reporter gene assays. Expression of ODC-E7 plus antizyme induced a >5-fold increase in E2F transcriptional activity in HEK293T cell lines (Figure 17B). In contrast, ODC-empty plus antizyme or ODC-E7 alone failed to activate E2F transcription activity. Therefore, ODC-E7 peptide affects endogenous Rb protein levels and E2F activity.

Effect of ODC-RANK peptide and ODC-TRAF6C on IL-1-induced NFκB reporter activity.

Previous data have suggested that TRAF6 may play an important role in IL-1-mediated NFκB activation but not in TNFα-mediated NFκB activation. Therefore, the effects of ODC-TRAF6C and ODC-RANK peptide on both IL-1- and TNFα-induced NFκB activation were examined.

To test the effect of ODC-RANK peptide and ODC-TRAF6C on IL-1-induced NFκB reporter activity, HEK293T cells were transiently transfected with a reporter gene plasmid (0.1 μg) that contains a NFκB responsive element cloned upstream of a luciferase reporter gene, together with 0.01 μg of pCMVβ-gal as a transfection-efficiency control, and 0.1 μg of plasmids encoding ODC, ODC-TRAF6C, ODC-RANK peptide, or Antizyme in various combinations, as indicated in Figure 18 (total DNA amount normalized). After 24 hours, cells were treated with 50 ng/ml IL-1 or 10 ng/ml TNFα for an additional 24 hours. Luciferase activity was measured in

cell lysates and normalized relative to β-galactosidase (mean ± std. dev.; n = 3).

As shown in Figure 18, expression of ODC-TRAF6C or ODC-RANK peptide in HEK293T cells induced marked reductions in NFκB reporter activity. In contrast, ODC-TRAF6C and ODC-RANK peptide did not affect the TNFα-madiated NFκB activation. These results confirm that TRAF6 is an important mediator in IL-1-induced signal transduction by using conditional inactivation of TRAF6 proteins.

A summary of the activity of various protein-degradation binding domains fused with ligands (target-protein binding domains) in the degradation of particular targets is shown in Table 5.

Table 5

Ligand	Target	ODC (N)	ODC+Az	SIP (N)	Siah (N)	E7 (C)	Fwb7 (C)	Ub1 (N)	Ub4 (N)	S5a (N)
TRFAF6-C	TRAF6	+	+	-	-	-	-	-	-	-
RANK-pep.	TRAF6	-	+	-	-	-	-	-	-	-
CD40CT	TRAF2	-	-	nd	nd	nd	nd	nd	nd	-
I-TRAF	TRAF2	-	-	-	nd	nd	nd	nd	nd	-
IKKα(LZ)	IKKα	-	-	-	nd	nd	nd	nd	nd	-
IKKβ(LZ)	IKKβ	+	+	-	nd	nd	nd	nd	nd	-
E7	Rb	-	+	-	-	nd	-	-	-	-
Caspase9(CARD)	Apaf1	-	-	-	-	-	-	-	-	nd
Apaf1(CARD)	Caspase9	nd	-	-	-	-	-	-	-	nd
FADD(DED)	Caspase8	nd	-	-	-	-	-	-	-	nd
BAG-1	HSP70	-	-	nd	nd	nd	nd	nd	nd	nd
p21	Cdk2	+	+	nd	-	-	nd	nd	nd	nd
Success Ratio	All Targets		5/12	0/9	0/7	0/6	0/6	0/6	0/6	0/7

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

Summary of Sequences

SEQ ID NO:1 is a cDNA (and the deduced amino acid sequence) encoding a Siah 1α of the present invention.

SEQ ID NO:2 is the deduced amino acid sequence of a Siah 1α protein of the present invention encoded by SEQ ID NO:1.

SEQ ID NO:3 is a cDNA (and the deduced amino acid sequence) encoding a human SIP-L polypeptide of the present invention.

SEQ ID NO:4 is the deduced amino acid sequence of a human SIP-L protein of the present invention encoded by SEQ ID NO:3.

SEQ ID NO:5 is a cDNA (and the deduced amino acid sequence) encoding a human SIP-S polypeptide of the present invention.

SEQ ID NO:6 is the deduced amino acid sequence of a human SIP-S protein of the present invention encoded by SEQ ID NO:5.

SEQ ID NO:7 is a cDNA (and the deduced amino acid sequence) encoding a human SAF-1α polypeptide of the present invention.

SEQ ID NO:8 is the deduced amino acid sequence of a SAF-1α protein of the present invention encoded by SEQ ID NO:7.

SEQ ID NO:9 is a cDNA (and the deduced amino acid sequence) encoding a human SAF-1β polypeptide of the present invention.

SEQ ID NO:10 is the deduced amino acid sequence of a SAF-1β protein encoded by SEQ ID NO:9.

SEQ ID NO:11 is a cDNA (and the deduced amino acid sequence) encoding a human SAF-2 polypeptide of the present invention.

SEQ ID NO:12 is the deduced amino acid sequence of a SAF-2 protein encoded by SEQ ID NO:11.

SEQ ID NO:13 is a cDNA (and the deduced amino acid sequence) encoding a human SAD polypeptide of the present invention.

SEQ ID NO:14 is the deduced amino acid sequence of a SAD protein encoded by SEQ ID NO:13.

SEQ ID NO:50 is the amino acid sequence of the ODC-fusion protein.